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1 **Medium-term effects of Ag is supplied directly or via sewage sludge to an agricultural**
2 **soil on *Eisenia fetida* earthworm and soil microbial communities.**

3
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26 **Abstract**

27

28 The widespread use of silver nanoparticles (AgNPs) in consumer products that release
29 Ag throughout their life cycle has raised potential environmental concerns. AgNPs primarily
30 accumulate in soil through the spreading of sewage sludge (SS). In this study, the effects of
31 direct exposure to AgNPs or indirect exposure via SS contaminated with AgNPs on the
32 earthworm *Eisenia fetida* and soil microbial communities were compared, through 3 scenarios
33 offering increasing exposure concentrations. The effects of Ag speciation were analyzed by
34 spiking SS with AgNPs or AgNO₃ before application to soil. SS treatment strongly impacted
35 Ag speciation due to the formation of Ag₂S species that remained sulfided after mixing in the
36 soil. The life traits and expression of *lysenin*, *superoxide dismutase*, *cd-metallothionein* genes
37 in earthworms were not impacted by Ag after 5 weeks of exposure, but direct exposure to Ag
38 without SS led to bioaccumulation of Ag, suggesting transfer in the food chain. Ag exposure
39 led to a decrease in potential carbon respiration only when directly added to the soil. The
40 addition of SS had a greater effect on soil microbial diversity than the form of Ag, and the
41 formation of Ag sulfides in SS reduced the impact of AgNPs on *E. fetida* and soil
42 microorganisms compared with direct addition.

43 **Keywords:** Silver nanoparticles, silver sulfide, ecotoxicology, earthworms, microorganisms,
44 speciation

45

46 **1. Introduction**

47 Silver nanoparticles (AgNPs) are widely used in various industries due to their unique
48 properties (Vance et al., 2015). The use of AgNPs in common consumer products leads
49 indirectly to environmental contamination (McGillicuddy et al., 2017). From manufacture to
50 the end of life of nano-functionalized products, the release of silver (Ag) in wastewater is
51 significant. Wastewater treatment plants (WWTPs) remove approximately 90% of AgNPs from
52 influents as sewage sludge (SS) (Kaegi et al., 2011; Ma et al., 2014; Tiede et al., 2010). Treated
53 SS is a biosolid rich in nutrients and organic matter and can be applied to agricultural soils as
54 fertilizer according to the circular economy aims of policymakers (European commission,
55 2017). Currently, there are no regulatory thresholds or recommendations for the Ag content of
56 SS used as fertilizer, and the ecotoxicity of Ag amendments to soil has not been fully
57 characterized. Based on previous studies and gaps in the field, Courtois et al. (2019) noted that
58 the risk associated with environmental contamination by Ag is poorly understand because of
59 the complexity of the soil matrix and the chemical transformations of AgNPs.

60 The present study sought to link silver speciation with its impact on the earthworm
61 *Eisenia fetida*, an important soil fauna test species in ecotoxicology, and on the natural
62 microflora. Earthworms are widely studied among soil invertebrates because they play a key
63 role in most continental ecosystems and represent an important part of the soil macrofauna.
64 They are considered “soil engineers” (Carbonell et al., 2009) that participate in the maintenance
65 of soil structure and fertility. In addition to enriching the soil with organic matter available for
66 plants, earthworms contribute to soil aeration and promote water penetration by forming
67 galleries during their movements (Bernard et al., 2010; Carbonell et al., 2009). Several studies
68 have shown that direct exposure to Ag in soil does not affect earthworm survival when the
69 concentration does not exceed a few tens of mg kg⁻¹ (Courtois et al., 2019). However,
70 reproduction is more sensitive to Ag exposure (Diez-Ortiz et al., 2015; Novo et al., 2015;

71 Schlich et al., 2013), and different stress markers have been observed (Gomes et al., 2015;
72 Hayashi et al., 2013; Shoults-Wilson et al., 2010). Exposure to the same concentrations of Ag
73 in SS appears to have reduced effects on earthworms compared with direct exposure, but only
74 two studies have addressed this issue (Lahive et al., 2017; Velicogna et al., 2017), without
75 verifying the speciation of Ag.

76 Soil microorganisms are key players in several ecosystem services. They participate in
77 soil fertility and stability via their roles in numerous biogeochemical cycles and degradation of
78 contaminants. Soil microorganisms also influence crop health via competition with pathogens
79 (Vance et al., 2015). Given the biocidal action of AgNPs, their effects on microorganisms are
80 of major concern. Several studies have reported decreases in the abundance and activities of
81 microorganisms and changes in the diversity of microbial communities in response to direct
82 exposure depending on the dose of AgNPs (Courtois et al., 2019; Hänsch and Emmerling, 2010;
83 He et al., 2016; Kumar et al., 2014; Liu et al., 2017; McGee et al., 2017; Samarajeewa et al.,
84 2017; Sillen et al., 2015). However, a much different pattern of response was observed after
85 exposure to AgNPs supplied via SS, with no or weak effects on the microbial communities
86 (Asadishad et al., 2018; Doolette et al., 2016; Durenkamp et al., 2016).

87 The main aim of this study was to assess the ecotoxicity of Ag introduced into the
88 environment through SS land spreading. To create a more realistic scenario, we performed
89 controlled lab-scale anaerobic digestion of Ag-spiked SS collected from a WWTP that was
90 subsequently spread at realistic doses on fresh agricultural soil. *Eisenia fetida* earthworms and
91 soil microorganisms were then subsequently exposed for 5 weeks to : 1) soil spiked with AgNPs
92 (with or without SS); 2) soil spiked with AgNO₃ (with or without SS); 3) control soil spiked
93 with the dispersant used to disperse AgNPs (with or without SS); and 4) control soil without
94 any additive (with or without SS).

95 The novelty of our study is the combined analysis of the behavior of different key
96 organisms in soil (earthworms and microorganisms) and Ag speciation to understand the
97 underlying mechanisms of AgNP toxicity. The specific goals of this research were as follows:

98 1) To assess the impact of various Ag species on earthworms' life traits (mortality, body
99 weight gain/loss, reproduction, expression of selected genes);

100 2) To assess the impact of various Ag species on soil microbial diversity and soil carbon
101 respiration activity;

102 3) To understand how chemical transformation of Ag in SS influences Ag toxicity
103 compared with direct exposure.

104

105 **2. Materials and methods**

106 ***2.1 Earthworm test species***

107 Genetically identified *E. fetida* earthworms (Homa et al., 2015) were obtained from a
108 laboratory breeding facility (LGCgE, University of Lille), where they were fed cow manure *ad*
109 *libitum*. Sub-adult earthworms were randomly selected, individually weighed, and introduced
110 into the microcosms. The earthworms weighed 296 mg on average (min: 104 mg, max: 751 mg,
111 standard deviation: 95 mg).

112

113 ***2.2. Soil***

114 Natural soil was collected in winter (January 2017) a few days before the beginning of
115 the experiment. The soil was a slightly calcareous (presence of chalk granules) brown soil
116 developed on wind-blown silts on chalky substrate, shallow to deep (DRAAF, 2013), from the
117 Haut de France Region (France, GPS coordinates: 50°59'94.87, 3°15'04.75). The collection
118 site has been used for vegetable production via certified organic agriculture since 2010, with no
119 use of pesticides or chemical fertilizers in the last 10 years. For the study, the first 20 cm layer

120 was collected and sieved at 5 mm. The metal content of the soil was as follows: 2.57 ± 1.97 mg
121 kg^{-1} Ag, 19.97 ± 0.35 mg kg^{-1} Cu, 387.67 ± 0.15 mg kg^{-1} Mn, 1.30 ± 1.19 mg kg^{-1} Ni, $49.67 \pm$
122 17.18 mg kg^{-1} Pb, and 70.90 ± 0.87 mg kg^{-1} mg kg^{-1} Zn. Cd was below the detection level (0.5
123 mg kg^{-1}).

124

125 **2.3. Sewage sludge**

126 Four WWTPs situated in southern Poland were pre-selected in order to monitor the
127 content of Ag during a two-year period. Among the four facilities, the facility that produced SS
128 with the lowest level of contamination and a preferable C:N ratio was selected. According to a
129 previous study, SS from the selected WWTP is a good source of nutrients for earthworms
130 without inducing stress related to the presence of contaminants ((Suleiman et al., 2017)). The
131 selected WWTP (Poland, GPS coordinates: $50^{\circ}55'22.81$ $19^{\circ}07'10.41$) is a small-sized plant that
132 uses activated sludge technology to support an agricultural area (flow: 1,000, population
133 equivalents: 20,000). The metal content of the SS was as follows: 11.53 ± 1.43 Ag, 0.95 ± 0.22
134 mg kg^{-1} As, 1.10 ± 0.24 mg kg^{-1} Cd, 140.62 ± 22.55 mg kg^{-1} Cr, 145.43 ± 37.38 mg kg^{-1} Cu,
135 186.78 ± 59.13 mg kg^{-1} Mn, 19.11 ± 9.02 mg kg^{-1} Ni, 32.21 ± 5.86 mg kg^{-1} Pb, and $2510.36 \pm$
136 615.99 mg kg^{-1} Zn (average based on measurements between March and December 2016).
137 Before agricultural reuse, anaerobic stabilization of the SS was performed at laboratory scale.

138

139 **2.4. Silver species**

140 The standard reference material Ag NM300K from the European Commission Joint
141 Research Centre (JRC), which has been fully characterized (Klein et al., 2011), was used as the
142 AgNPs in this study. The NPs were spherical and corresponded to a colloidal dispersion with a
143 nominal Ag content of 10.2% by weight, dispersed in 4% w/w% each of polyoxyethylene
144 glycerol trioleate and polyoxyethylene sorbitan mono-laurate. The nominal size of 99% of the

145 particles was approximately 15 nm without coating, and transmission electron microscopy
146 (TEM) indicated a size of 17 ± 8 nm. Smaller nanoparticles of approximately 5 nm were also
147 present (Mendes et al., 2015). The commercial nanoparticle NM300K was kindly provided by
148 the Fraunhofer Institute for Molecular Biology and Applied Ecology IME. Each bottle
149 contained 2 g of NM300K, which was diluted in dispersant with a volume of 2 mL; the resulting
150 solution contained 10% (w/w) Ag (0.1 g of Ag per 1 mL). AgNO₃ solution was prepared by
151 dissolving AgNO₃ salt in sterile distilled water. The solutions of AgNPs and Ag ions (from
152 AgNO₃) were both diluted with milliQ water to obtain a Ag concentration of approximately 2
153 mg mL⁻¹.

154

155 **2.5. Experimental scheme**

156 *2.5.1. Anaerobic digestion of sewage sludge*

157 A batch anaerobic digestion of SS was performed in parallel in four continuous stirred-
158 tank bioreactors. In the first bioreactor, SS was introduced without any additives (AD-control).
159 In the second, SS was spiked with 40 mg L⁻¹ NM300K AgNPs (AD-AgNPs). In the third, only
160 a corresponding quantity of dispersant (AD-dis) was added to the SS, and in the fourth, 40 mg
161 L⁻¹ AgNO₃ (AD-AgNO₃) was added. The selected Ag concentration in the bioreactors was
162 based on the maximum concentration of Ag that does not disturb anaerobic fermentation (Yang
163 et al., 2012) (Full justification in SI 1)

164 The bioreactors were glass vats filled with 6 L of SS maintained under mesophilic
165 conditions at a temperature of 37 °C with constant mixing (180 rpm) using a mechanical stirrer.
166 Details of the equipment as well as the methods of analyzing pH, volatile fatty acids, volatile
167 solids, total solids, and ammonium nitrogen were described previously (Grosser, 2017). After
168 4 weeks of anaerobic digestion (AD), the process had stabilized; the bioreactors were then
169 stopped, and the digestates were centrifuged at 12100 rcf for 15 minutes.

170

171 *2.5.2. Microcosm exposure - experimental mixtures*

172 Earthworms and soil microbial communities were exposed in microcosms subjected to
173 8 treatments (at three different concentrations) in triplicate over 5 weeks (Figure SI 2).

174

175 a) Four of the treatments corresponded to indirect exposure, i.e., mixed with SS ("AD-X").

176 A realistic application quantity of SS was introduced to the microcosms in a single addition. In
177 France, the maximum dose of SS spreadable over 10 years (Circular DE / GE n ° 357 of
178 03/16/99, 1999) was divided by 10, and quantities equivalent to 3, 6 and 10 times this calculated
179 quantity were applied. The details of the selection of these quantities of SS for addition to the
180 microcosms are given in SI 3. The three different dosages of SS were as follows: 60 g of fresh
181 SS per kg of fresh soil as the 3-year perspective, "perspective 3" (3y); 120 g kg⁻¹ as the 6-year
182 perspective, "perspective 6" (6y); and 200 g kg⁻¹ for the 10-year perspective, "perspective 10"
183 (10y). The amount of Ag remaining in the SS after the fermentation process was estimated as
184 0.233 mg of Ag per g of fresh SS. Thus, the estimated amount of Ag was 14, 28 and 47 mg kg⁻¹
185 (fresh matter) for perspectives 3, 6, and 10, respectively (details of this estimation are provided
186 in SI 4). The mean humidity of soil mixtures was 21 % therefore, the estimated amount of Ag
187 in dry mixtures was 18, 35 and 59 mg kg⁻¹ (dry matter). The microcosms were filled with 1 kg
188 of these mixtures.

189 - AD-AgNPs condition: soil supplied with SS digested with AgNPs in dispersant (AD-AgNPs-
190 3y, AD-AgNPs-6y, AD-AgNPs-10y);

191 - AD-dis: soil supplied with SS digested with dispersant solution consisting 4% w/w% each of
192 polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan mono-laurate (AD-dis-3y,
193 AD-dis-6y, AD-dis-10y);

194 - AD-AgNO₃: soil supplied with SS digested with AgNO₃ (AD-AgNO₃-3y, AD-AgNO₃-6y,
195 AD-AgNO₃-10y);

196 - AD-control: soil supplied with SS digested without any addition (AD-control-3y, AD-control-
197 6y, AD-control-10y).

198

199 b) Four treatments corresponded to direct exposure (i.e., without addition of SS). As in the
200 microcosms with SS, 14, 28 and 47 mg kg⁻¹ (fresh matter) of Ag was added to perspectives 3,
201 6, and 10, respectively, but this Ag was added directly to the soil.

202 - AgNPs: soil supplemented with a solution of AgNPs in dispersant (AgNPs-3y, AgNPs-6y,
203 AgNPs-10y);

204 - Dis: soil supplemented with the corresponding volume of dispersant (Dis-3y, Dis-6y, Dis-
205 10y),

206 - AgNO₃: soil supplemented with AgNO₃ solution (AgNO₃-3y, AgNO₃-6y, AgNO₃-10y)

207 - Control: soil without any addition.

208

209 The mixtures were prepared as described above and distributed into plastic boxes with
210 perforated lids (18 x 18 x 9 cm), 1 kg of fresh matter per box. Twelve earthworms were
211 introduced per microcosm. The soil and earthworms were analyzed before the experiment and
212 after 5 weeks of exposure. A subsample of fresh soil was stored at -18 °C before microbial
213 DNA extraction and at 4°C for microbial respiration analysis.

214

215 **2.6. Analysis**

216 2.6.1. Biological analysis

217 *Life traits of earthworms: reproduction, survival and biomass*

218 Reproduction potential was estimated by counting cocoons and juveniles at the end of the
219 exposure period and observing the viability of the collected cocoons. Survival was measured
220 by counting earthworms that survived exposure. The biomass of the groups of earthworms was
221 measured before and after exposure. To compensate for the initial differences in biomass
222 between the microcosms, the final biomass value was expressed as a percentage.

223

224 *Gene expression levels in earthworms*

225 Coelomocytes of earthworms were collected by extrusion as described previously (Brulle et al.,
226 2006). Extrusion is a non-invasive method (Diogène et al., 1997; Eyambe et al., 1991) that
227 involves electrical stimulation of earthworms in a cold environment. Stress causes the expulsion
228 of coelomocytes by nephridial pores. Then, RNA was extracted from the coelomocytes
229 following the Tri-Reagent® protocol (Molecular Research Center, USA). Reverse transcription
230 was performed using 1.5 µg of RNA with the Omniscript RT kit (Qiagen, Netherlands), RNase
231 inhibitor (Thermo Fisher Scientific, USA) and primers (Invitrogen and Thermo Fisher
232 Scientific, USA). Quantitative PCR were performed with the MESA Blue qPCR MasterMix
233 Plus for SYBR® Assay no ROX kit (Eurogentec, France). The qPCR conditions were as
234 follows: denaturation at 95°C for 5 min, 40 cycles of amplification and extension (each cycle
235 comprising 3 sec at 95°C, 30 sec at 60°C and 10 sec at 72°C), a melting curve step (progressive
236 heating from 60 to 95°C), and then cooling to reach 40°C. Two previously validated reference
237 genes were used: *β-actin* (Forward 5'-GTACGATGAGTCCGGG-3' and 5'-
238 GCATGTGTGTGTGGTGTC-3') and the *ribosomal protein S13* (Forward 5'-
239 CGCACGGTTTTAGTTTCT-3' and Reverse 5'-CCATGCGAGTCTCGAAG-3') (Bernard et
240 al., 2010). The gene encoding β-actin (an intracellular eukaryotic protein) is the most commonly
241 used housekeeping gene for qPCR quantification. The gene encoding ribosomal protein S13
242 (RPS13) has also been used previously as a housekeeping gene (Rorat et al., 2017). Three target

243 genes were tested: *superoxide dismutase (sod)* (Forward 5'-GGCGATAACACAAATGGT-3'
244 and Reverse 5'-CGTGCGTCCAATGATTGAA-3'), *lysenin (lys)* (Forward 5'-
245 CGGCAACAAACGTCTAC-3' and Reverse 5'-GTGAAATACAGGCAGAAG-3') and *cd-*
246 *metallothionein (cdmt)* (Forward 5'-CGCAAGAGAGGGATCAACTT-3' and Reverse 5'-
247 CTATGCAAAGTCAAAGTCTGTC-3'). These genes are often used as biomarkers in earthworms.
248 The *sod* gene is associated with oxidative stress (Choi and Park, 2015). The SOD protein
249 catalyzes the destruction of hydrogen peroxide, a molecule created during oxidative stress that
250 is dangerous for cells (Bernard et al., 2015). The *lysenin* gene is required for the synthesis of
251 the hemolytic protein LYS, which is involved in immunity and associates with sphingomyelin
252 to permit membrane pore formation (Bernard et al., 2010). LYS is a key protein in the secretome
253 of coelomocytes (Hayashi et al., 2015). Finally, the *cdmt* gene plays a role in early defense
254 against toxic metal ions and oxidative stress (Hayashi et al., 2013); this gene is a good
255 biomarker of exposure to heavy metals because its expression increases during exposure to Cd
256 in particular but also other metallic elements (Höckner et al., 2011). The reactions were
257 performed using a LightCycler®480 with LightCycler Software (Roche Diagnostics, France).
258 The geometric mean of the two reference genes was used (Brulle et al., 2006). The relative
259 expression of each gene of interest was calculated using the formula of Pfaffl (Livak and
260 Schmittgen, 2001): $R = 2^{-(C_{Ptarget} - C_{Ppref})}$. The induction factor corresponds to $R_{treatment}/R_{associated}$
261 treatment without silver.

262

263 *Microbial community composition*

264 DNA was extracted from 500 mg of sieved soil using a PowerSoil® DNA Isolation kit
265 (Qiagen, France) according to the manufacturer's instructions. The V4–V5 hypervariable region
266 of the 16S rRNA gene targeting *Bacteria* and *Archaea* was amplified using the primers 515F
267 (5'-GTGYCAGCMGCCGCGGTA-3') and 928R (5'-CCCCGYCAATTCMTTTRAGT-3').

268 The reaction mixture included 1.40 μL of each primer (20 μM each), 28 μL of AmpliTaq Gold
269 Master mix (AmpliTaq Gold; 360 Master Mix Applied Biosystems), 2 μL of template DNA at
270 a concentration of 5 $\text{ng } \mu\text{L}^{-1}$ and water qsp 55 μL . The cycle conditions included initial
271 denaturation at 94 $^{\circ}\text{C}$ for 10 min, followed by 35 cycles of denaturation at 95 $^{\circ}\text{C}$ for 30 s,
272 annealing at 65 $^{\circ}\text{C}$ for 30 s and extension at 72 $^{\circ}\text{C}$ for 40 s and an additional extension step at
273 72 $^{\circ}\text{C}$ for 10 min after cycling was complete. Illumina MiSeq sequencing was performed using
274 the 2x250 paired-end protocol with an Illumina[®] MiSeq instrument at the GeT plage facility,
275 Toulouse, France (<http://get.genotoul.fr>).

276

277 *Potential carbon respiration*

278 The potential carbon respiration rate is also called substrate-induced respiration and is
279 measured under optimal conditions of temperature and substrate (Anderson and Domsch,
280 1978). Briefly, fresh soil equivalent to 10 g of oven-dried soil was placed in a sterile 150 ml
281 plasma flask with a rubber stopper. One milliliter of glucose was added to obtain a final
282 concentration of 3 mg glucose g^{-1} dry soil. Additional water was added to achieve 70% of the
283 water holding capacity. The plasma flasks were closed and incubated at 28 $^{\circ}\text{C}$ for 3.5 h. Gas
284 samples were analyzed at 0, 1, 2, 3, and 3.5 h for CO_2 concentration using a gas chromatograph
285 (P200 Micro, Agilent Technology, Massy, France).

286

287 2.6.2. Physicochemical analysis

288 *Metal content in earthworms and soils*

289 On day zero of exposure, some earthworms from the breeding facility were sacrificed to
290 measure the quantities of metals present in their bodies. After Ag exposure, earthworms from
291 each microcosm were sacrificed for the same purpose. Before sacrifice, a depuration phase of
292 24 h was conducted in order to empty the intestinal content. The organisms were sacrificed by

293 freezing for at least 48 h and then lyophilized by group from the same microcosm for
294 approximately 60 h. The samples were ground to a powder using liquid nitrogen and then
295 mineralized by acid digestion (using HNO₃, H₂SO₄ and HCl₄) at high temperature as described
296 by Bernard et al. (9). The resulting solution was analyzed by ICP-OES (inductively coupled
297 plasma-optical emission spectrometry) (Varian 720-ES, USA) to quantify Cd, Co, Cu, Mn, Ni,
298 Pb, Zn and Ag.

299 Soil samples, SS samples and mixed samples were collected and lyophilized at the
300 beginning and end of exposure. The dried samples were ground with a mortar and pestle. For
301 mineralization, 300 mg of sample was digested in 7 mL of concentrated HNO₃ using a Berghof
302 microwave digestion system (speed wave MWS-2-Microwave pressure digestion). The
303 resulting solution was analyzed by ICP-OES (Thermo apparatus) to quantify Cd, Co, Cu, Mn,
304 Ni, Pb, Zn, and Ag.

305

306 *Speciation of silver in earthworms and soils*

307 Samples of earthworms and the mixtures of soil and SS were analyzed to determine the
308 chemical state of Ag. Ag speciation was determined by X-ray absorption spectroscopy. Ag K-
309 edge (25.514 keV) XANES (X-ray absorption near-edge structure) spectra were acquired at the
310 European Synchrotron Radiation Facility (ESRF, France) on the FAME beamline (BM30b)
311 with Si(220) monochromator crystals ((Proux et al., 2005)).

312 Prior to analysis, the samples were lyophilized, ground and pressed into 5 mm pellets.
313 Spectral acquisition was performed at liquid helium temperature to avoid sample evolution
314 under the beam. Measurements were carried out in fluorescence mode using a 30-element
315 Canberra Ge solid-state detector. Each spectrum was the sum of at least three scans. A set of
316 model compounds including metallic Ag (AgNPs), Ag-humic acids (Ag-HA), Ag₂S, Ag-
317 thiocarbamate (Ag-thiocarb) and Ag-glutathione (Ag-GSH) (the last two corresponding to

318 thiolated compounds linked to Ag) was run in transmission mode. Normalization and data
319 reduction were performed according to standard methods using Athena software ((Ravel and
320 Newville, 2005)).

321 The residual factor of linear combination fitting was calculated as follows: $R = \frac{\sum(\text{exp} - \text{fit})^2}{\sum(\text{exp})^2}$, where the sums are over the data points in the fitting region. At each step of the
322 fitting, an additional reference spectrum was added if the following two conditions were true:
323 the R factor decreased by 20% or more and the additional reference had a contribution equal to
324 or greater than 10% among Ag species.

326

327 ***2.7. Bioinformatics analysis of microbial communities***

328 Sequence analysis was performed with the pipeline FROGS from the Galaxy portal of
329 the Toulouse Midi-Pyrenees bioinformatics platform (Escudié et al., 2018). After a
330 preprocessing step that included quality filtering, read trimming and read assembly, the
331 sequences were clustered with Swarm (Mahé et al., 2014) with an aggregation distance of 3 and
332 a denoising clustering step. Chimeras were removed using VSEARCH (Rognes et al., 2016)
333 combined with original cross-sample validation. Operational taxonomic units (OTUs) with
334 abundances lower than 0.005% were removed (Bokulich et al., 2013). SILVA database 128
335 (release date 29.09.2016) was used to perform the OTU affiliations (Quast et al., 2013). In order
336 to compare samples, a normalization procedure was performed with random resampling down
337 to 14,165 sequences.

338

339 ***2.8 Statistical analysis***

340 The majority of the earthworm biomass, mortality, gene expression and metal content
341 data did not follow a normal distribution, and the variances were not homogeneous between
342 treatments (Shapiro-Wilk, Lilliefors and Bartlett tests). Thus, Sheirer-Ray-Hare nonparametric

343 tests and post-hoc tests based on ranks were used. When data fulfilled statistical assumption of
344 parametric tests (normality and homogeneity of variances), analysis of variance (ANOVA) and
345 Tukey HSD (honestly significant difference) post-hoc tests were used. Correlation matrices
346 (based on the Kendall method) were constructed.

347 For microorganism analyses, one-way ANOVA and Tukey tests were performed with
348 the software PAST (Hammer et al., 2001) to determine if there were significant differences in
349 respiration between the treatments. Microbial community composition was analyzed using
350 PRIMER software (PRIMER-E Ltd., Plymouth, UK). Dissimilarity in OTU composition
351 between all pairs of microbial communities was computed using Bray-Curtis distance and
352 nonparametric permutational multivariate analysis of variance (PerMANOVA) (Anderson,
353 2001) was conducted to test for difference in composition among treatments. PerMANOVA
354 was performed using permutation tests with 9,999 iterations. Results of the PerMANOVAs
355 were visualized using non-metric multidimensional scaling ordinations (NMDS) based on
356 Bray-Curtis distances. Finally, an indicator analysis (IndVal) (Dufrene and Legendre, 1997)
357 was carried out to estimate the degree of association between OTUs and treatments. The
358 analysis quantifies the fidelity and specificity of species (OTU) in relation to treatments or to
359 groups of treatments, i.e., with or without SS on one hand and with or without Ag on the other
360 hand, and tests for the statistical significance of the associations using permutation tests with
361 9,999 iterations (De Cáceres et al., 2010; Dufrene and Legendre, 1997). The indicator value of
362 species i for class j is obtained using the equation $IndVal_{ij} = 100 \cdot A_{ij} \cdot B_{ij}$, where A_{ij} is specificity,
363 i.e., the proportion of the individuals of the species i that are in the class j , and B_{ij} is fidelity,
364 i.e., the proportion of sites in the class j that contain the species i .

365 Unless otherwise mentioned, statistical analyses were implemented within the R programming
366 environment (R Core Team, 2008).

367

368 **3. Results**

369 **3.1. Biological analysis**

370 3.1.1. Life traits: reproduction, survival and biomass

371 The percentage of survival of earthworms after exposure are presented in Table 1.
372 Statistical tests showed no significant differences between any of the treatments and their
373 respective controls without Ag (i.e. the control of AgNPs is Dis, for AgNO₃ it is Control, for
374 AD-AgNPs it is AD-dis and for AD-AgNO₃ it is AD-control). Ag did not cause mortality under
375 these conditions. A significant impact of SS addition was observed in treatment AD-X-10y
376 (perspective 10) with or without Ag. The addition of the highest dose of digested SS led to the
377 death of all earthworms living in the microcosms.

378 *Table 1: Mean percentage of survival in the microcosms. Standard deviations are indicated.*

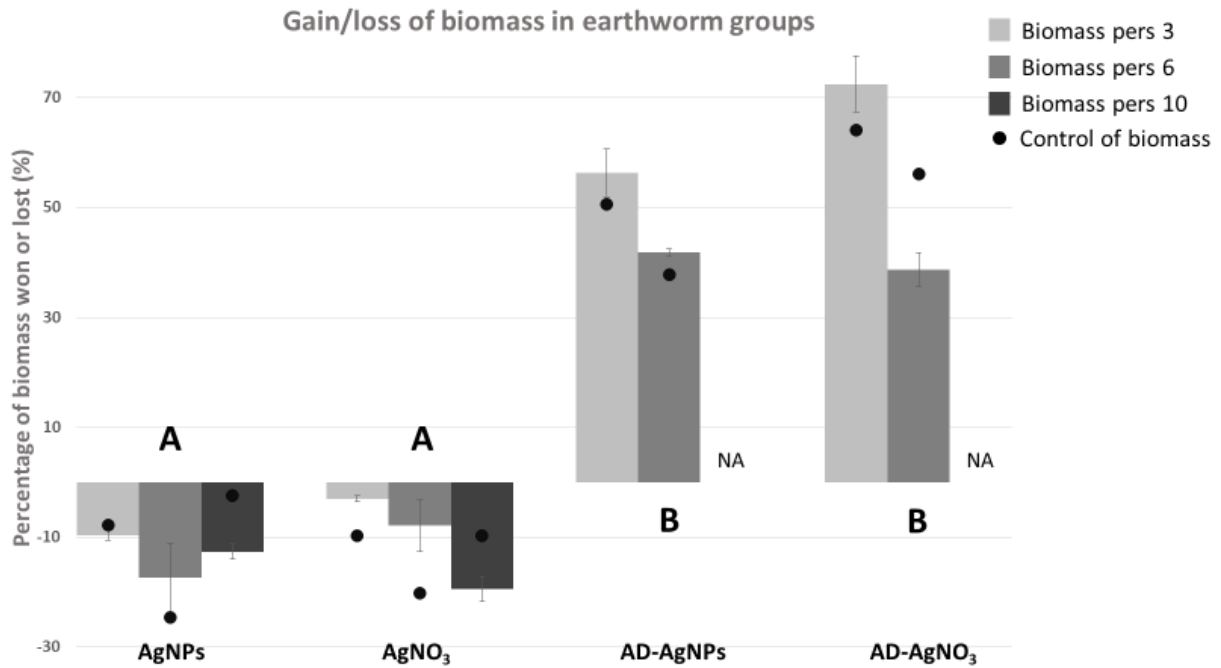
	Survival % in Perspective 3	Survival % in Perspective 6	Survival % in Perspective 10
Control	100.0 ± 0.0	97.2 ± 4.8	100.0 ± 0.0
Dis	100.0 ± 0.0	100.0 ± 0.0	97.2 ± 4.8
AgNPs	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
AgNO ₃	100.0 ± 0.0	100.0 ± 0.0	91.7 ± 14.4
AD-control	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
AD-dis	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
AD-AgNPs	100.0 ± 0.0	95.8 ± 5.9	0.0 ± 0.0
AD-AgNO ₃	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0

379
380 From the second week of exposure, cocoons were observed in certain microcosms, and from
381 the 4th week, juveniles were born. The reproduction was also not affected by Ag, regardless of
382 its chemical form, since there was no significant difference between the number of cocoons or
383 juveniles in Ag treatments compared to respective controls without Ag (Table 2). However,
384 there were significant differences between the treatments with SS and those without SS. Almost
385 no reproduction was observed in the microcosms without SS, while in the microcosms with SS,
386 breeding was significant, and the cocoons were viable.

387 *Table 2: Number of cocoons and juveniles in the microcosms. Standard deviations are indicated. Empty cells in the table*
 388 *corresponds to data no available because of the high mortality in theses conditions.*

	Cocoons			Juveniles		
	Perspective 3	Perspective 6	Perspective 10	Perspective 3	Perspective 6	Perspective 10
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	0 ± 0
Dis	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	0 ± 0
AgNPs	0 ± 0	1 ± 1	0 ± 0	0 ± 0	2 ± 2	0 ± 0
AgNO ₃	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 1	0 ± 0
AD-control	13 ± 7	3 ± 1	-	5 ± 2	0	-
AD-dis	7 ± 2	18 ± 3	-	5 ± 2	2	-
AD-AgNPs	6 ± 2	10 ± 2	-	3 ± 2	1 ± 1	-
AD-AgNO ₃	32 ± 12	15 ± 9	-	5 ± 2	3 ± 1	-

389
 390 The changes in earthworm biomass observed during exposure are presented in Figure 1.
 391 In all treatments, body weight gain or loss was not significantly different from that in the
 392 respective control treatments. Thus, no effect of Ag (nano or ionic, added directly or via
 393 fermented SS) on earthworm biomass was observed. However, there were some significant
 394 differences between treatments. Earthworms exposed to treatments without SS lost up to 31%
 395 of body weight showing a stress, while worms exposed to treatments with SS gained
 396 considerable weight (between 34 and 176%).
 397



398

399 *Figure 1: Percentage of weight gain or loss in the groups of earthworms in the microcosms between the beginning and end of*
 400 *the experiment (percentages are reported to compensate for differences in initial weight). Each perspective is represented by*
 401 *a color: light grey for perspective 3, medium grey for perspective 6 and dark grey for perspective 10. The black dots correspond*
 402 *to the mean earthworm biomass under control conditions (dispersant for AgNPs, control for AgNO₃, AD-dis fir AD-AgNPs*
 403 *and AD-control for AD-AgNO₃). NA corresponds to non-available data due to the high mortality in these conditions. The big*
 404 *letters show the differences between conditions AgNPs, AgNO₃, AD-AgNPs and AD-AgNO₃ (all perspectives combined). There*
 405 *were no differences between perspectives within the same condition.*

406

407 3.1.2. Gene expression levels in earthworms

408 The analysis of the crossing-points (Cp) of *actin* and *RS13* genes showed that actin was
 409 very stable for all conditions and perspective. RS13 was very stable for all conditions in
 410 perspective 3 and 10, however, for the perspective 6, RS13 was less stable. Therefore, results
 411 of 3 target genes were analyzed in two ways: with both reference genes as all other data and
 412 with only one reference gene (Actin) like in the publications of Brulle et al. (2011) and Homa
 413 et al. (2015). The obtained results were similar.

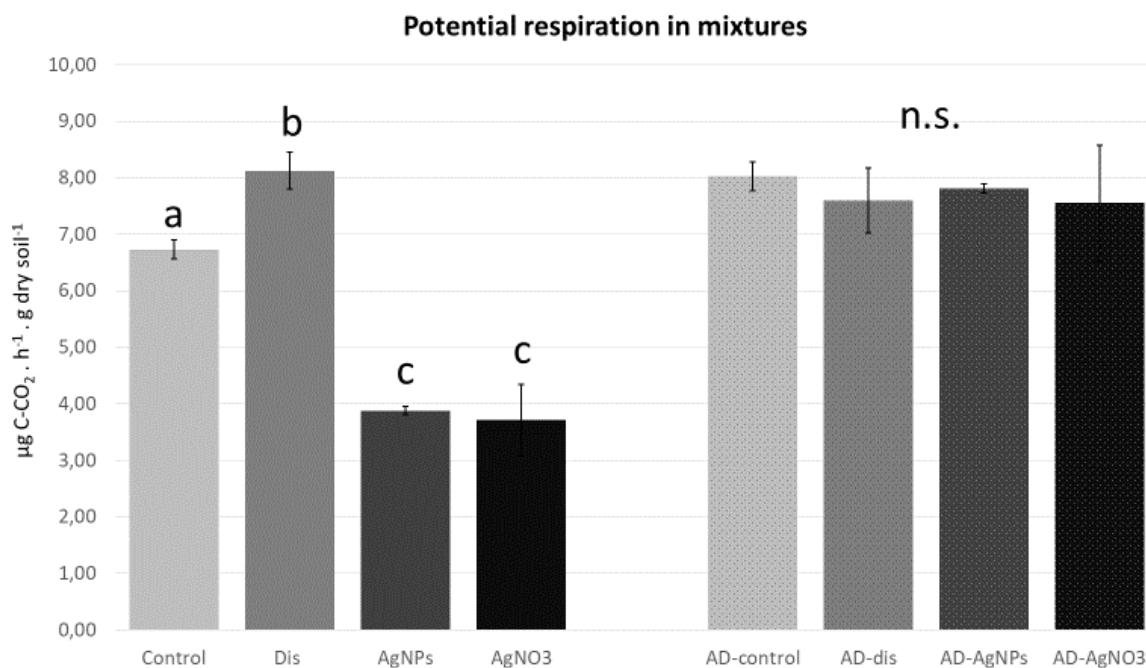
414 The analysis of gene expression showed no significant variations in the 3 target genes: *lys*, *sod*
415 and *cdmt* (Figures SI 5, SI 6 and SI 7). SS and Ag did not induce the transcription of these 3
416 genes for these 3 concentrations.

417

418 3.1.3 Potential carbon respiration

419 All microorganism activity and diversity measurements were performed only for
420 perspective 3. After direct exposure to Ag (AgNPs or AgNO₃) in soil, a strong and significant
421 decrease in potential carbon respiration was observed compared to the control and dispersant-
422 treated soils. There was no effect of Ag form initially added in soil because the impact of AgNPs
423 and AgNO₃ was similar on the potential respiration. No effect on potential carbon respiration
424 was observed when Ag (AgNPs or AgNO₃) was applied on soil via SS (Figure 2), with a
425 positive effect of SS application on microbial carbon respiration.

426



427

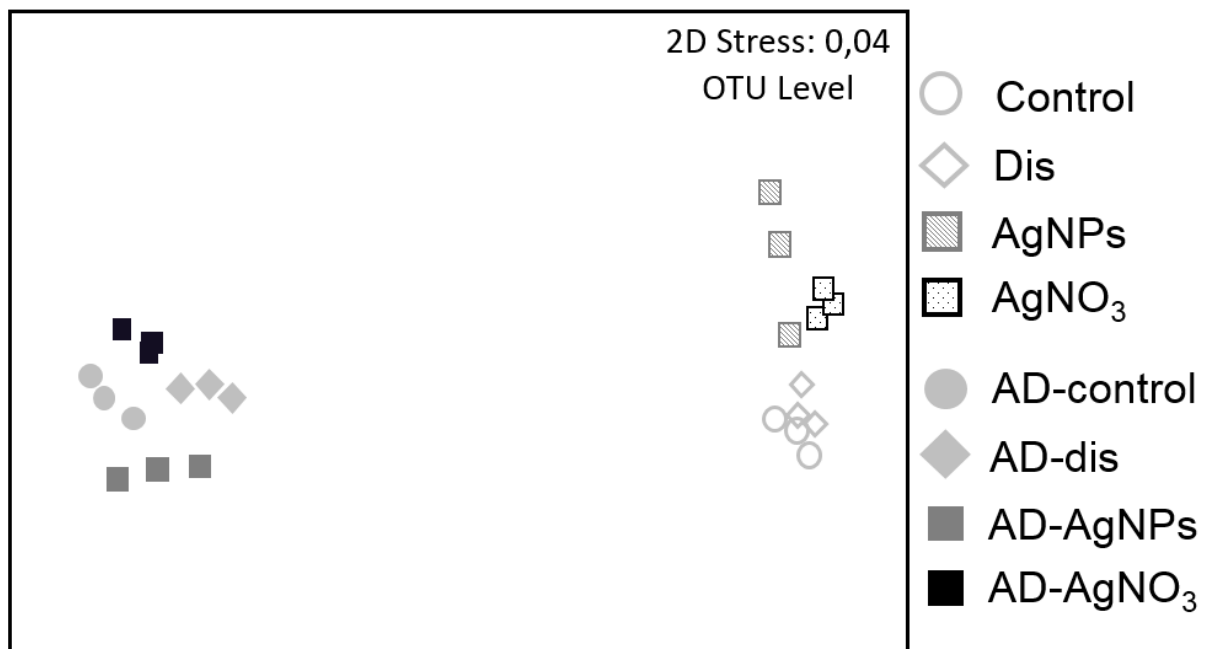
428 Figure 2: Potential carbon respiration of microbial communities in mixtures (µg C-CO₂ · h⁻¹ · g⁻¹ of dry soil). The letters indicate
429 significant differences in respiration between the 4 treatments without sewage sludge. “n.s.” indicates that there were no
430 significant differences between the 4 treatments with sewage sludge.

431

432 3.1.4. Microbial community composition

433 After 5 weeks of the experiment, microbial community composition differed greatly
434 depending on the presence or absence of SS. The addition of SS explained 52% of the variance
435 between these 2 groups (Figure 3). Ag addition (with or without SS) explained another 12% of
436 the variance. Interactions between routes of entry (direct exposure or via SS application) and
437 Ag form (AgNPs or AgNO₃) explained 12% of the variance.

438



439

440 *Figure 3: Two-dimensional ordination solution using non-metric multidimensional scaling (NMDS) based on the Bray-Curtis*
441 *distance showing the relative proximities of microbial community composition among all treatments.*

442

443 The IndVal analysis (based on 519 OTU) failed to extract any indicator of Ag exposure at the
444 species or genus level (Table SI 8). However, the Indval analysis based on samples supplied
445 with or without SS led to identify 118 microorganisms indicating of SS supply. Among them,
446 we found 9 Archaea from the *Methanobacteriales* and *Methanomicrobiales* orders and 109
447 bacteria from families and orders often found in anaerobic environments and gastrointestinal

448 tract of different animals. For instance, we identified 22 members of *Clostridiales* all known as
 449 obligate anaerobes and several representatives of the *Porphyromonadaceae*, *Rikenellaceae*
 450 families.

451

452 3.2. Physicochemical analysis

453 3.2.1. Metal content in mixtures

454 The soil concentrations of Ag were lower than expected (Table 3). The average soil Ag
 455 concentrations in perspectives 3, 6 and 10 were $11.56 \pm 1.69 \text{ mg kg}^{-1}$, $16.97 \pm 3.99 \text{ mg kg}^{-1}$ and
 456 $25.38 \pm 3.69 \text{ mg kg}^{-1}$, respectively. In perspectives 3 and 10, the Ag concentrations in AD-
 457 AgNPs, AD-AgNO₃, AgNPs and AgNO₃ were similar. In perspective 6, there was a slight
 458 difference in Ag concentrations between the treatments with direct addition of Ag and those
 459 with addition of Ag via SS.

460

461 Table 3: Silver concentration in mixtures (mg kg⁻¹ of dry mixture). Results were obtained by ICP analysis. Standard deviations
 462 are indicated. Some standard deviations are missing due to a lack of replicates.

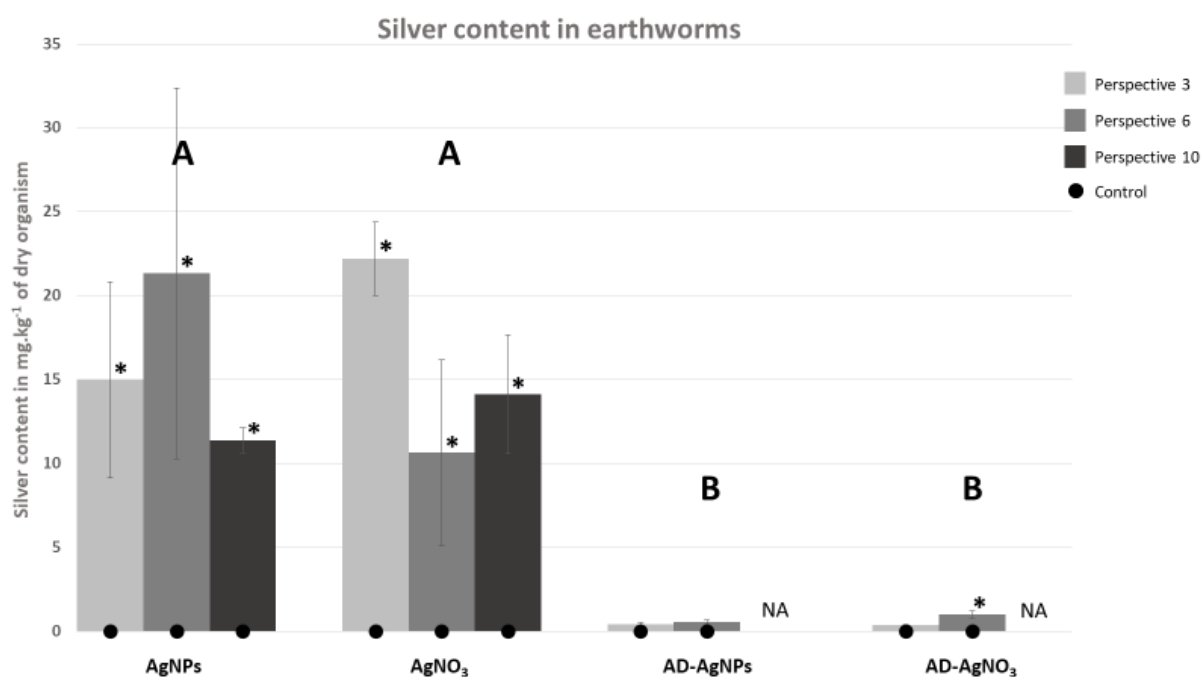
Condition	Perspective 3	Perspective 6	Perspective 10
AD-control	2.00 ± 0.61	1.71 ± 0.23	4.57 ± 0.86
AD-AgNPs	9.54 ± 0.52	15.00 ± 1.56	23.77 ± 1.48
AD-dis	3.69 ± 0.80	2.15	8.36 ± 0.24
AD-AgNO ₃	11.63 ± 0.23	12.60	27.97 ± 5.25
AgNPs	11.23 ± 0.84	17.9 ± 1.95	27.40 ± 2.55
Dis	2.55 ± 0.35	0.45 ± 0.41	0.94 ± 0.87
AgNO ₃	13.83 ± 0.78	22.37 ± 1.76	22.37 ± 2.20
Control	0.09 ± 0.15		

463

464 3.2.2. Metal content in earthworms

465 The contents of 7 metals in earthworms were analyzed after exposure (Table SI 9). Since the
 466 organisms exposed to SS in perspective 10 did not survive, metal content data were not
 467 available for these microcosms. No significant variation in Pb and Zn was observed regardless
 468 of the treatment and perspective. Cd, Cu, and Mn were bioaccumulated differently by the
 469 earthworms depending on exposure to SS, with greater bioaccumulation in the presence of SS.
 470 However, there was no impact of Ag on the bioaccumulation of these metals. The
 471 concentrations of Cu and Mn in worms increased with their concentrations in the soil (presence
 472 or absence of SS in the microcosms). Conversely, the bioaccumulation of Cd was lower in the
 473 presence of higher concentrations of Cd in the soil.
 474 In all treatments without Ag addition, the Ag content in earthworms was below the detection
 475 level. Bioaccumulation of Ag in earthworms was significant when they were exposed to
 476 microcosms directly contaminated with Ag (Figure 4). In microcosms with SS application, Ag
 477 bioaccumulation in earthworms was significant only in the condition AD-AgNO₃-6y and was
 478 detectable in AD-AgNPs-3y, AD-AgNPs-6y and AD-AgNO₃-3y.

479



480

481 *Figure 4: Silver content in earthworms (mg of Ag kg⁻¹ of dry matter). Each perspective is represented by a color: light grey for*
482 *perspective 3, medium grey for perspective 6 and dark grey for perspective 10. NA means data are non-available due to the*
483 *high mortality in these conditions. The big letters indicate significant differences among conditions AgNPs, AgNO₃, AD-AgNPs*
484 *and AD-AgNO₃ (no difference between perspectives within the same condition). The black dots correspond to the mean biomass*
485 *of earthworms in the control (dispersant for AgNPs and control for AgNO₃). The stars (*) indicate significantly different values*
486 *compared with the associated control without silver.*

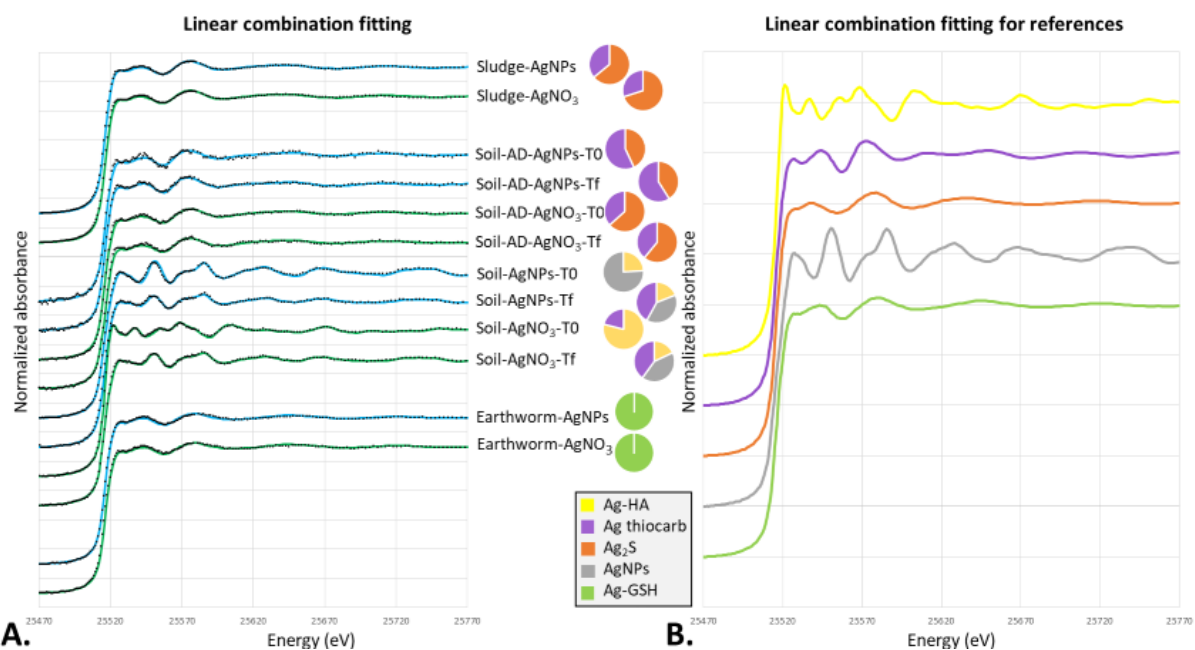
487

488 3.2.3. Speciation of silver in soil

489 *AgNPs and AgNO₃ in soil*

490 In the absence of SS, initially (approximately 2 h after spiking), the speciation of Ag
491 varied depending on its initial form (Figure 5). Ag initially from AgNPs was mainly in its
492 pristine form (76% metallic Ag), with the remainder bound to organic matter (24%). This latter
493 fraction was probably the fraction of soluble Ag that quickly dissolved and was complexed by
494 the organic matter present in the soil. The Ag initially from AgNO₃ was quickly complexed
495 with organic matter with a fraction of thiol groups (21% Ag-thiol). Importantly, after the 5
496 weeks of the experiment, Ag speciation no longer depended on the initial speciation. In the
497 presence of either AgNPs or AgNO₃, approximately 40% of Ag was in the metallic form, 40%
498 was complexed to an organic thiol, and 20% was bound to humic acids.

499



500 **A.** Speciation of silver in sewage sludge, soils and mixtures at the beginning (T0) or end (Tf) of the soil incubations
 501 and speciation of silver in earthworms: linear combination fitting of XANES spectra is shown on the left (dotted lines
 502 correspond to experimental data, and solid colored lines correspond to the fits). Samples (soils, mixtures, sewage sludge or
 503 earthworms) from conditions with AgNPs are in blue. Samples from conditions with AgNO₃ are in dark green. **B.** XANES
 504 spectra of Ag references. Ag-HA (in yellow) corresponds to Ag complexed with humic acids. AgNPs (in gray) corresponds to
 505 the linear combination fitting obtained with a sample of NM300K AgNPs used for the experiment. Ag₂S (in orange) corresponds
 506 to acanthite, a silver sulfide. Ag-thiocarb (in purple), and Ag-GSH (in light green) corresponds to Ag linked to a thiol-
 507 containing organic compound.

509
 510 *AD-AgNPs and AD-AgNO₃ in soil*

511 When spiked in SS prior to addition to soil, the Ag in the SS was transformed into Ag₂S
 512 (30-33%) and Ag bound to a thiol-containing organic compound (67-70%), regardless of the
 513 initial state of the Ag (ionic or NPs) (Figure 5). These two species remained the main species
 514 after the addition of SS to the soil, with small variations in the relative proportions of the two
 515 species. The speciation of Ag did not change after the 5 weeks of the experiment.

516
 517 3.2.4. Speciation of Ag bioaccumulated in earthworms

518 Investigation of Ag speciation in earthworms was possible only for the soil incubated
519 without SS since the content of bioaccumulated Ag in earthworms exposed to the AD-AgNPs
520 and AD-AgNO₃ microcosms was too low to be analyzed by X-ray absorption spectroscopy
521 (Figure 4). The speciation of Ag bioaccumulated in earthworms was similar regardless of the
522 initial form of Ag when exposed to AgNPs or AgNO₃. The XANES spectra were identical to
523 those of the Ag-thiol model compound (Figure 5).

524

525 **4. Discussion**

526 Regardless of chemical form, concentration and direct/indirect exposure scenario, Ag
527 had no impact on the life traits of *E. fetida* relative to the controls. At the investigated doses,
528 Ag did not cause earthworm death and did not affect the reproduction or the biomass of the
529 groups of earthworms. Changes in life traits were observed only for the addition of SS. SS is
530 an important source of organic matter (Suleiman et al., 2017), and thus the food resources
531 available for earthworms were greater in the microcosms supplemented with SS, which allowed
532 the earthworms to gain weight and reproduce. Conversely, the earthworms in the microcosms
533 without SS lost weight and stopped reproducing. These results in conditions without SS show
534 that the worms were stressed, possibly due to a lack of food, so this is important to keep this in
535 mind for the overall results. The absence of addition of food was intentional in order to avoid
536 bringing other contaminants that would prevent comparisons of microcosms with and without
537 SS. Also, adding a large amount of SS, such as in perspective 10, had drastic consequences for
538 earthworm survival. It is known that SS sometimes contains a lot of ammonium, a very toxic
539 compound (Rorat, 2015).

540 The accumulation of Ag by earthworms did not depend on the Ag concentration in the
541 soil or the initial form of Ag (NPs or ionic). Direct supply of Ag (AgNPs or AgNO₃) led to
542 significant bioaccumulation of Ag in earthworms (10–20 mg kg⁻¹), whereas the addition of the

543 two forms of Ag via SS only resulted in slight bioaccumulation (2 mg kg⁻¹ max). Thus, Ag in
544 SS was less bioavailable for earthworms. It is possible that the difference in consumption of the
545 substrate attenuated / exaggerated these differences in bioaccumulation due to the different MO
546 contents between the microcosms with and without SS, however this difference in
547 bioavailability linked to Ag speciation is a result already known for several animal and plant
548 species (Pradas del Real et al., 2016; Velicogna et al., 2017). Differences in the bioaccumulation
549 of Cd, Cu, and Mn in earthworms were observed depending on exposure to Ag. However, the
550 content of metals was dependent on the presence or absence of SS rather than the presence of
551 Ag. SS contains a cocktail of pollutants and metals (including Cd, Cu and Mn) at high
552 concentrations compared with normal soil.

553 Regardless of its form and concentration, the presence of Ag did not alter the expression
554 of lysenin (*lys*), superoxide dismutase (*sod*) and cadmium metallothionein (*cdmt*) genes in
555 earthworms after 5 weeks of exposure. These genes, which are involved in oxidative stress
556 (Choi and Park, 2015), immunity (Hayashi et al., 2015) and defense against toxic metal ions
557 (Hayashi et al., 2013), have been studied previously in the context of Ag. According to the
558 literature, AgNPs and AgNO₃ at high concentration (500 mg kg⁻¹) in natural soil does not affect
559 the expression of the *sod* gene (Hayashi et al., 2013) while in artificial soil, an overexpression
560 of *sod* has been observed with 100 mg kg⁻¹ of AgNO₃ only (Choi and Park, 2015). In this last
561 cited study, AgNPs did not affect the *sod* gene expression, even at low concentration (1 to 100
562 mg kg⁻¹). Only one study showed the effect of Ag on the *lys* gene expression with in-vitro
563 conditions. Authors exposed coelomocytes of earthworms to solutions of AgNPs and AgNO₃
564 at low concentrations during 24 hours and saw a rapid upregulation (measure after 2 h of
565 exposure) of *lys* only this AgNPs and a late down-regulation (measure after 8 or 24 h of
566 exposure) (Hayashi et al., 2015). *Cdmt* gene has also been studied a little in the context of
567 exposure to Ag. Significant overexpressions of *cdmt* have been shown in several studies the

568 first few days (between 1st and 7th day) of exposure with Ag. In artificial and natural soils, *cdmt*
569 seems to be mainly affected by the high Ag contents (Choi and Park, 2015; Hayashi et al., 2013)
570 but in Curieses Silvana et al. (2017) low (0.05 mg kg⁻¹) and high concentrations caused this
571 upregulation. In some cases, when the exposure lasts longer (10 – 14 days), downregulation of
572 *cdmt* can be observed with low and medium concentrations (0.05 to 50 mg kg⁻¹) in artificial
573 soil (Bourdineaud et al., 2019; Curieses Silvana et al., 2017). In all these cited studies, the
574 measure of relative expression levels of these 3 genes were measured during the first 2 weeks
575 of exposure. Short-term and long-term defense mechanisms may vary, and it is possible that by
576 observing the expression of the genes after 5 weeks we missed earlier changes in the expression.

577 The results of this study showed that the Ag bioaccumulated in earthworms was bound
578 to organic thiols, consistent with a previous study (Baccaro et al., 2018) and observations in *E.*
579 *fetida* in a different context of exposure (Courtois et al., 2020). The localization of Ag in
580 organisms, its speciation, and Cu-Ag competition for bioaccumulation observed in the latter
581 studies, as well as studies on the role of metallothionein (MT) (Demuyne et al., 2006; Morgan
582 et al., 2004; Sugawara and Sugawara, 1984; Vijver et al., 2004) and changes in the expression
583 of MT-encoding genes in Ag exposure contexts (Curieses Silvana et al., 2017; Hayashi et al.,
584 2013), suggest that Ag in earthworms are linked to MT. Regardless of the Ag concentration in
585 soil (perspectives 3, 6, and 10), bioaccumulation in earthworms was similar, suggesting the
586 existence of a regulation mechanism to limit the accumulation and therefore the toxicity of Ag.
587 Such a regulation mechanism in earthworms might involve MT. Thus, by observing all
588 published results, it would seem that the metalloproteins play an early role (first few days /
589 weeks) in the detoxification of Ag (metallic or ionic) in *E. fetida*, hence an overexpression of
590 the gene at the start of exposure. Then the expression of the gene returns to normal, probably
591 because other defense mechanisms take over, which would explain that in the study below, at
592 5 weeks, no change in gene expression was observed.

593 With respect to soil microorganisms, a negative impact of direct exposure to both Ag
594 forms (NPs and ionic) on potential carbon respiration was observed after 5 weeks of the
595 experiment. However, no effects were detected when Ag was applied via SS. Potential carbon
596 respiration is carried out by numerous groups of optional or obligatory aerobic microorganisms.
597 The absence of an effect on potential carbon respiration does not imply that no microorganism
598 was affected by Ag but instead indicates that the whole community managed to compensate for
599 any negative effects, if any, of Ag on certain groups of microorganisms. The positive effect of
600 the dispersant on potential carbon respiration upon direct addition to the soil can be explained
601 by the fact that the dispersant is a polysorbate, a derivative of sorbitol that is metabolizable by
602 microorganisms. A similar effect was not observed when digested SS with dispersant was
603 added, perhaps because the dispersant had been fully used by the slime microorganisms during
604 anaerobic digestion.

605 The microbial community composition established using metagenomic tools differed
606 greatly between the microcosms treated with or without SS. It may be explained by changes in
607 microbial composition due to addition of nutrients in the SS. However, 140 microorganisms
608 indicating a SS supply were identified. Most of them are usually found in anaerobic
609 environments and gastrointestinal tract of different animals. This likely indicates the persistence
610 of DNA coming from anaerobic microorganisms present in SS. These microorganisms might
611 be dead or no longer active in soils where conditions are not favorable to them and can hide the
612 weaker effects of Ag supply on the microbial community composition. The microbial
613 composition in the control and dispersant treatments differed from that in the Ag treatments.
614 However, the majority of the variation of microbial composition was due to the addition of SS
615 rather than the addition of Ag. Thus, the differences in response between the two applications
616 modes showed that during SS digestion at the WWTP, Ag underwent strong transformations
617 that led to a total absence of effects on respiration activity and a slight impact on diversity.

618 Several authors have reported effects of direct exposure to AgNPs on microbial community
619 composition (Rahmatpour et al., 2017; Samarajeewa et al., 2017), but the design adopted in the
620 present experiment revealed no impact of Ag on the microbial community. The present results
621 are consistent with the few previous studies conducted under similar conditions (Doolette et al.,
622 2016; Durenkamp et al., 2016), which did not observe major effects of low doses of Ag that
623 had undergone fermentation on microbial abundance and communities. However, a short-term
624 effect of Ag cannot be excluded. Indeed, our experiments lasted 35 days, and thus effects
625 occurring within a few days or weeks followed by resilience would not be detected. No
626 microorganisms specifically resistant to Ag were observed, and therefore no microbial indicator
627 of this kind of contamination in soils can be used.

628 Thus, the lasting effects of introduction of Ag to the terrestrial environment via SS were
629 slight changes in the soil microbial community and slight but not necessarily significant
630 accumulation of Ag in earthworms. There was no effect of the form of Ag initially provided or
631 of the Ag concentration in the microcosms. Thus, the effects of Ag in SS were strongly
632 attenuated compared with direct introduction of Ag (2 forms) into the soil. These observations
633 can be explained by Ag speciation in soil. During the anaerobic fermentation of SS, Ag
634 underwent chemical transformations. Both AgNO₃ and AgNPs changed in the same manner,
635 and at the end of fermentation, the two forms of Ag were completely sulfided, consistent with
636 previous studies (Levard et al., 2012; Pradas del Real et al., 2016). After mixing in the soil, the
637 Ag remained completely sulfided. These chemical forms were stable and did not evolve much
638 during the 5 weeks of incubation. Silver sulfidation strongly decreases silver toxicity to a
639 variety of (micro-)organisms due to the high chemical stability of the Ag-S species (Levard et
640 al., 2013, 2011; Reinsch et al., 2012). Likewise, when introduced directly into soil, the
641 speciation of AgNO₃ and AgNPs evolved in a similar manner by the end of 5 weeks (with not
642 more than 40% of Ag bound to sulfur molecules). Thus, in both modes of supply, AgNPs and

643 AgNO₃ ultimately had the same speciation and therefore similar effects on *E. fetida* and soil
644 microorganisms. Finally, at low doses, Ag sulfides are less bioavailable than Ag⁺ or AgNPs to
645 certain organisms (Courtois et al., 2019), like earthworms (Lahive et al., 2017). The SS initially
646 added with AgNPs and AgNO₃ essentially contained Ag sulfides at the end of fermentation.
647 Anaerobic treatment of SS thus reduces the toxicity of Ag to organisms.

648

649 **5. Conclusion**

650 In conclusion, after 5 weeks, no strong effect of Ag on the microbial community and
651 earthworms was observed when Ag was supplied to the soil via a reasonable quantity of SS.
652 Speciation did not differ between AgNPs and AgNO₃ after the major chemical changes that
653 occurred during fermentation. Interestingly, the microbial communities seemed to be highly
654 resistant to Ag species supplied with sewage sludge, and the earthworms seemed to accumulate
655 much less Ag due to reduced bioavailability. The effects of Ag observed in the absence of SS
656 were strongly limited when Ag was previously sulfided due to the digestion of SS.

657 The results of this study need to be confirmed in other soil types, as microflora are highly
658 site-specific. Other types of SS treatments and shorter time scales should also be investigated
659 to exclude immediate effects (on microorganisms as well as earthworms), and longer time
660 scales should be evaluated to assess the effects of consecutive additions of SS. For earthworms,
661 it would be interesting to study the gene network linked to metallic stress to highlight the genes
662 potentially affected by Ag. Because bioaccumulation was observed, the potential for trophic
663 transfer of Ag should be explored. For microbial communities, processes that are more sensitive
664 than respiration, such as nitrification, could be analyzed. Respiration is performed by a large
665 number of microbial taxa, whereas nitrification is an activity supported by a small number of
666 microbial taxa. Effects of Ag on some of these latter taxa could greatly impact nitrification in
667 soil.

668

669

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681

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958

Object: Submission Chemosphere

Dear Editor,

Please find attached the manuscript entitled «**Medium-term effects of Ag is supplied directly or via sewage sludge to an agricultural soil on Eisenia fetida earthworm and soil microbial communities**» for consideration to publication as a Research article in Chemosphere. Information on the co-authors can be found at the end of this letter.

This article focus on the effects caused by silver contamination of agricultural soil due to human practices. Indeed, silver nanoparticles (AgNPs) used in consumer products end especially in soils via spreading of biosolids since wastewater treatment retains Ag in sewage sludge. Ecotoxicological assessment is very important since Ag can impact terrestrial environment and create ecological and economic problems. In this study, we assessed the effects of direct exposure to AgNPs or indirect exposure via sewage sludge contaminated with AgNPs on the earthworm test species *Eisenia fetida* and soil microbial communities. We have carried out various and numerous physico-chemical (speciation, dosages) and biological (life traits, gene expression, potential carbon respiration, microbial diversity) analysis.

We believe that this article would have a perfect place in Chemosphere journal because fit perfectly to your topics: emerging contaminant, environmental fate with bioaccumulation, speciation of contaminant, adverse effects of contaminant in terrestrial organisms and effects of nanoparticles in the environment. In addition, it could have a wide audience interested by environmental subjects. Indeed, this study is the only one which allows a global vision of the effects of the silver brought by sewage sludge in agricultural soil, bringing together biological, microbiological and chemical points of view, while respecting realistic contamination conditions. Here, the multidisciplinary does not prevent from showing complex and in-depth results in each area. In addition, we believe that this article will encourage new research since "realistic" studies in this area are largely missing. (see our recent Review published in Environmental Pollution by Courtois et al., 2019 ; <https://doi.org/10.1016/j.envpol.2019.07.053>).

Please, find below a list of names and addresses of referees familiar with this topic:

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We have carefully read the author guidelines and wrote this manuscript accordingly to meet the requirements of Chemosphere. Also, the paper has been reread and validated (spell and grammar) by a certified native English speaker. All co-authors have contributed and agree with its content. None of co-authors have any conflicts of interest with regards to this study and there is no financial interest to report. Also, we confirm that all data, figures, pictures and images in the graphical abstract and the manuscript were created by the co-authors. Finally, we certify that you are currently the only journal to which we are submitting this article, so it is not being reviewed in other journals. Part of the results were presented at conferences, with posters and oral platform, which do not spoil the originality of these data and discussion.

We thank you for taking time to consider this study and we look forward to hearing from you in the earliest convenience.

Yours sincerely,

Pauline COURTOIS

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1 **Medium-term effects of Ag is supplied directly or via sewage sludge to an agricultural**
2 **soil on *Eisenia fetida* earthworm and soil microbial communities.**

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Medium-term effects of Ag is supplied directly or via sewage sludge to an agricultural soil on *Eisenia fetida* earthworm and soil microbial communities.

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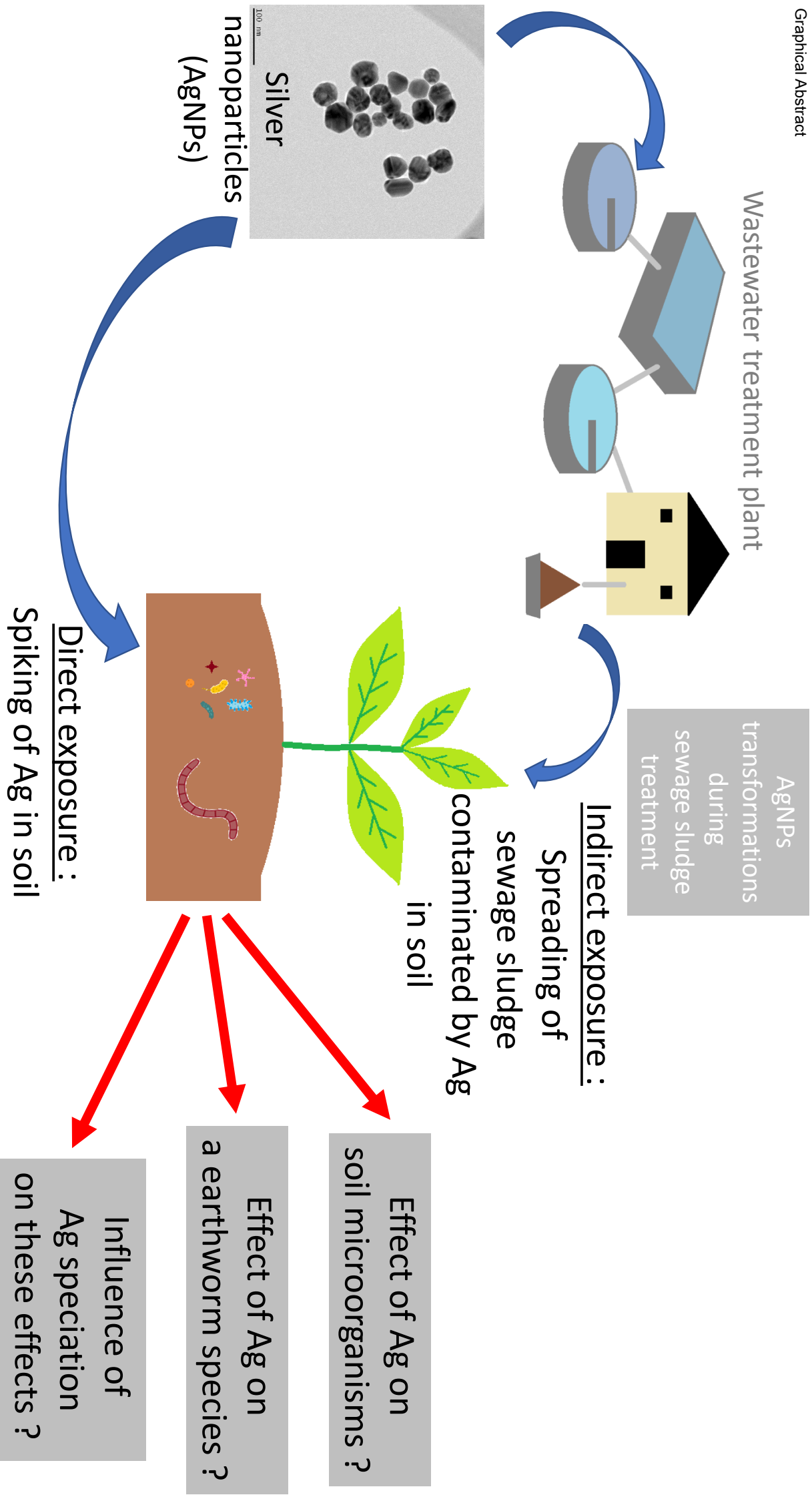
Highlights:

- Ag is brought to the agricultural soil from spreading of contaminated sewage sludge
- *Eisenia fetida* bioaccumulate few amount of Ag in realistic scenario of exposure
- In realistic scenario, Ag change a little the diversity of soil microbial communities
- Speciation of Ag in sewage sludge causes less effect than nanoparticulate metallic Ag

Object: Submission Chemosphere

Title: Medium-term effects of Ag is supplied directly or via sewage sludge to an agricultural soil on Eisenia fetida earthworm and soil microbial communities

None of co-authors have any conflicts of interest with regards to this study and there is no financial interest to report.



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8

9 **Abstract**

10 The widespread use of silver nanoparticles (AgNPs) in consumer products that release
11 Ag throughout their life cycle has raised potential environmental concerns. AgNPs primarily
12 accumulate in soil through the spreading of sewage sludge (SS). In this study, the effects of
13 direct exposure to AgNPs or indirect exposure via SS contaminated with AgNPs on the
14 earthworm *Eisenia fetida* and soil microbial communities were compared, through 3 scenarios
15 offering increasing exposure concentrations. The effects of Ag speciation were analyzed by
16 spiking SS with AgNPs or AgNO₃ before application to soil. SS treatment strongly impacted
17 Ag speciation due to the formation of Ag₂S species that remained sulfided after mixing in the
18 soil. The life traits and expression of *lysenin*, *superoxide dismutase*, *cd-metallothionein* genes
19 in earthworms were not impacted by Ag after 5 weeks of exposure, but direct exposure to Ag
20 without SS led to bioaccumulation of Ag, suggesting transfer in the food chain. Ag exposure
21 led to a decrease in potential carbon respiration only when directly added to the soil. The
22 addition of SS had a greater effect on soil microbial diversity than the form of Ag, and the
23 formation of Ag sulfides in SS reduced the impact of AgNPs on *E. fetida* and soil
24 microorganisms compared with direct addition.

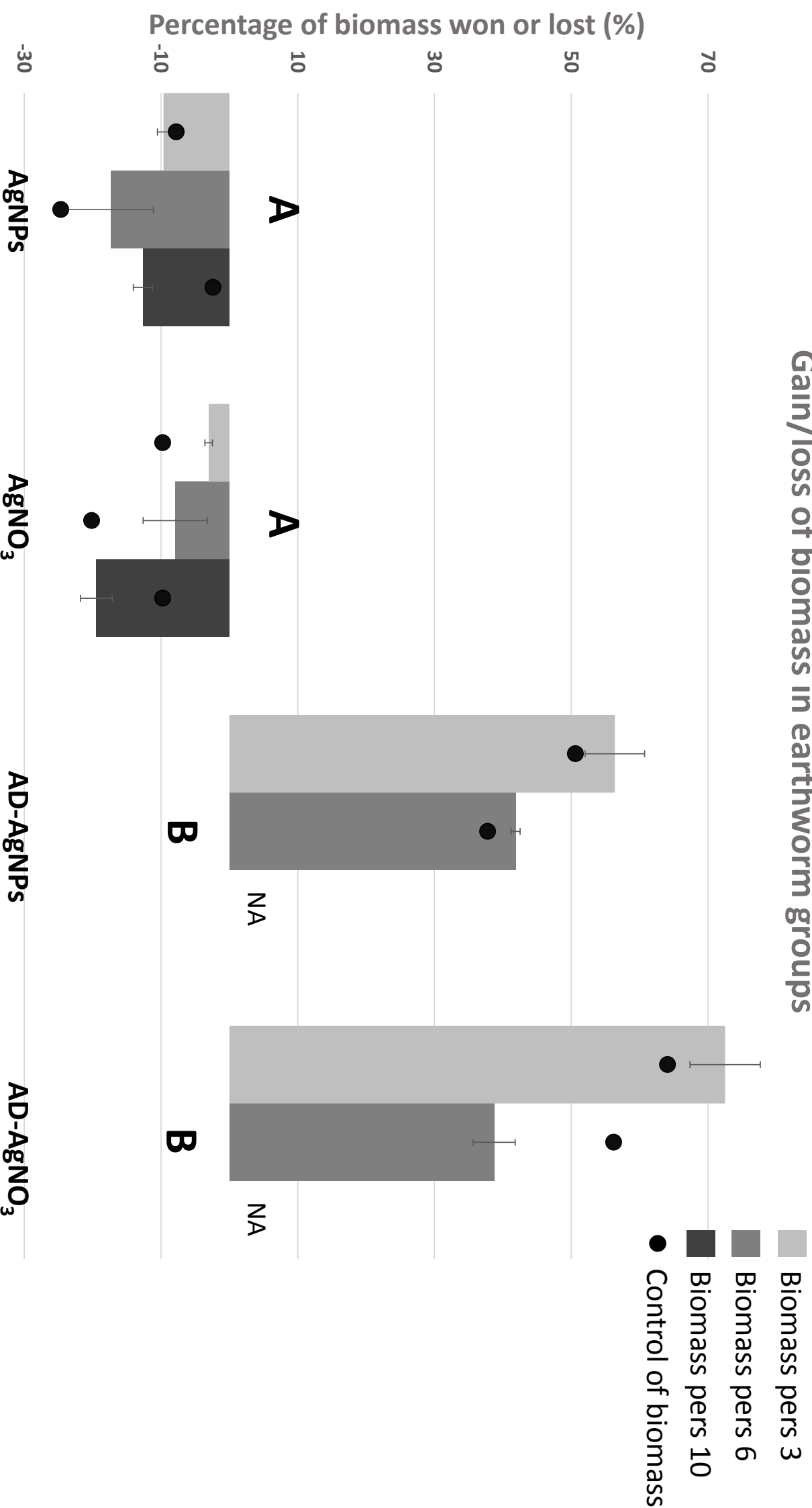
25

26 **Keywords:** Silver nanoparticles, silver sulfide, ecotoxicology, earthworms, microorganisms,
27 speciation

Figure 1

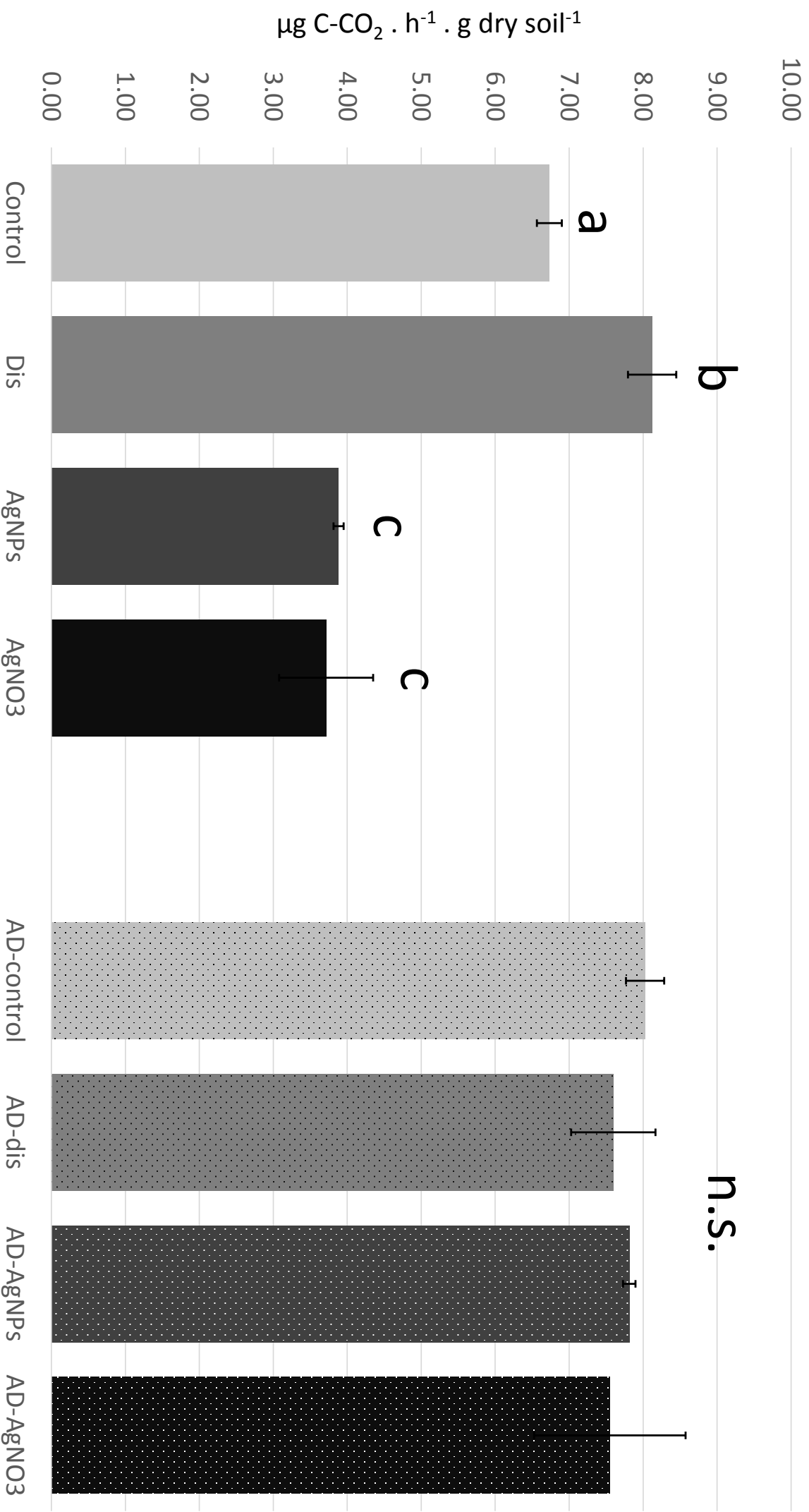
Gain/loss of biomass in earthworm groups

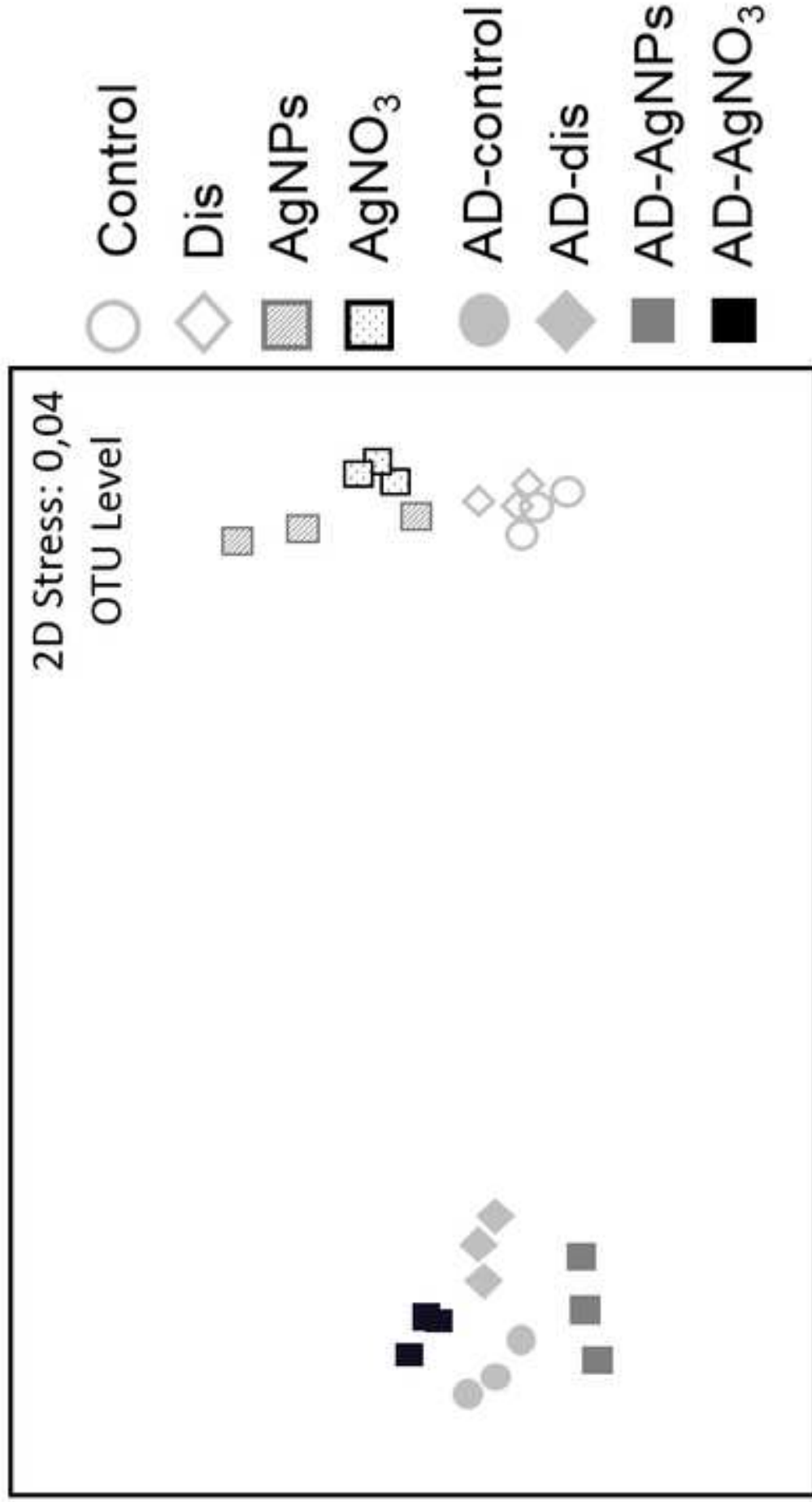
[Click here to access/download:Figure:Fig 1 biomass.pptx](#)



Potential respiration in mixtures

[Click here to access/download;Figure;Fig 2 respiration sols.pptx](#)





Silver content in earthworms

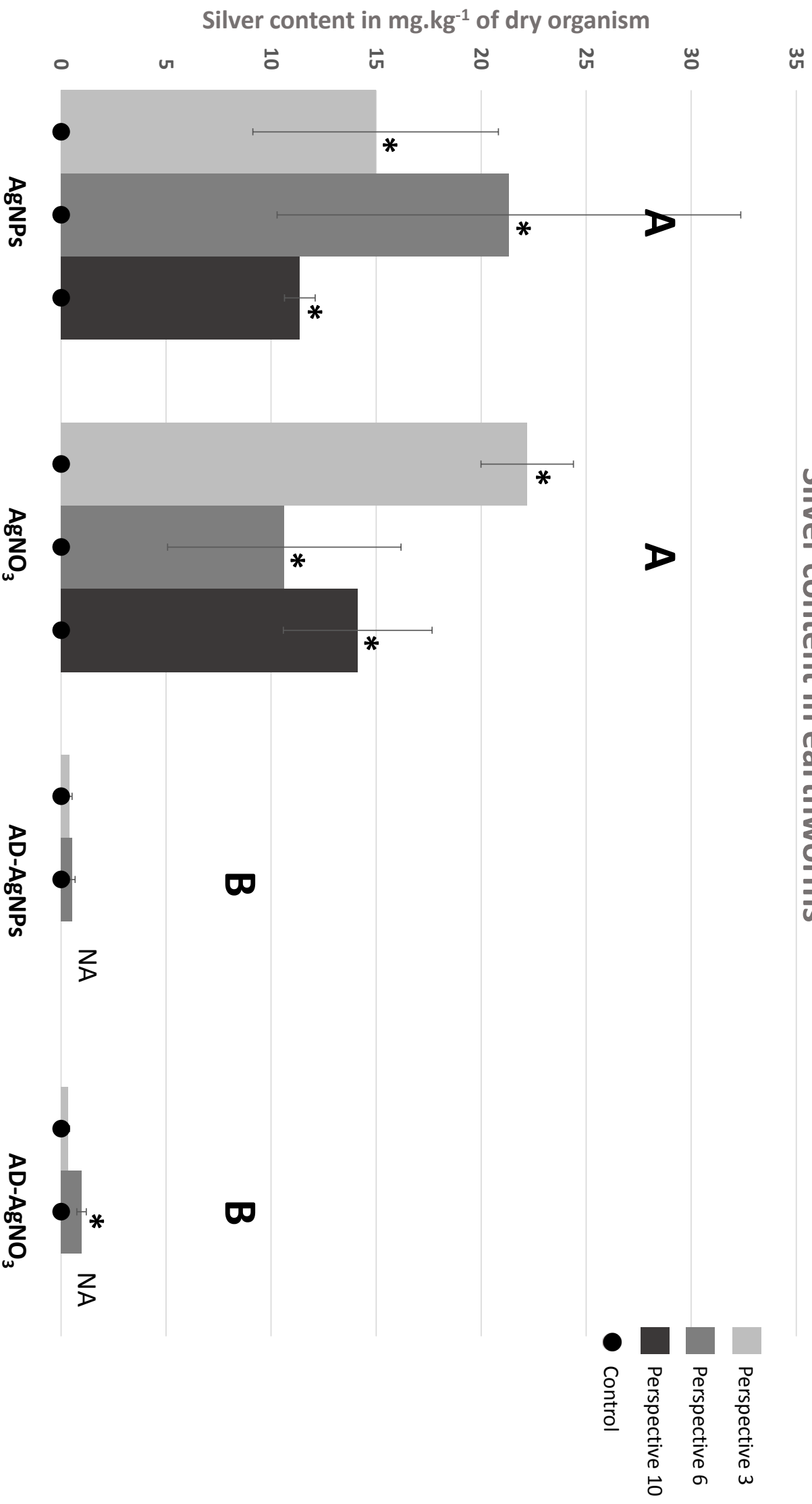
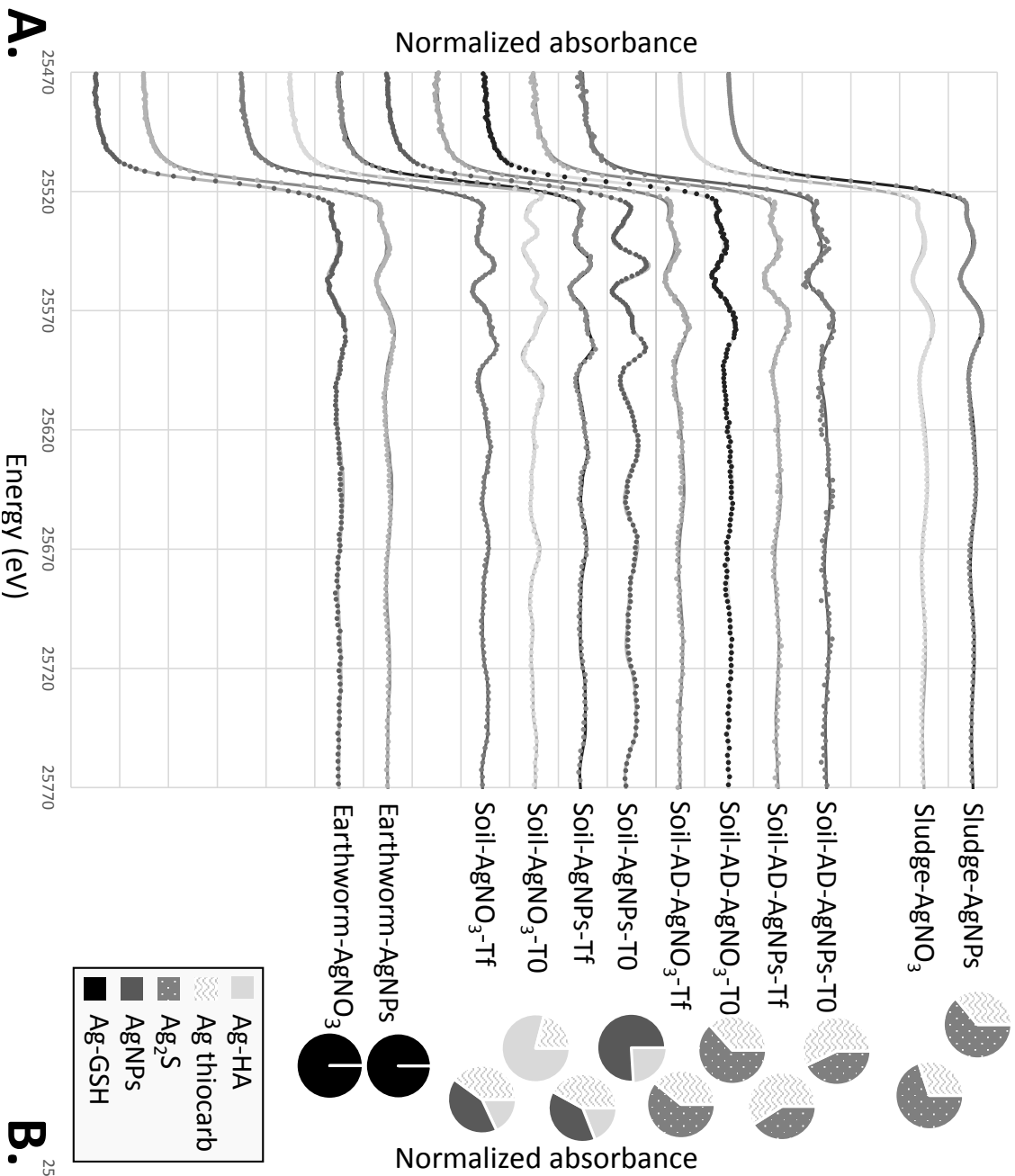


Figure 5 (colorless)

Linear combination fitting



Linear combination fitting for references

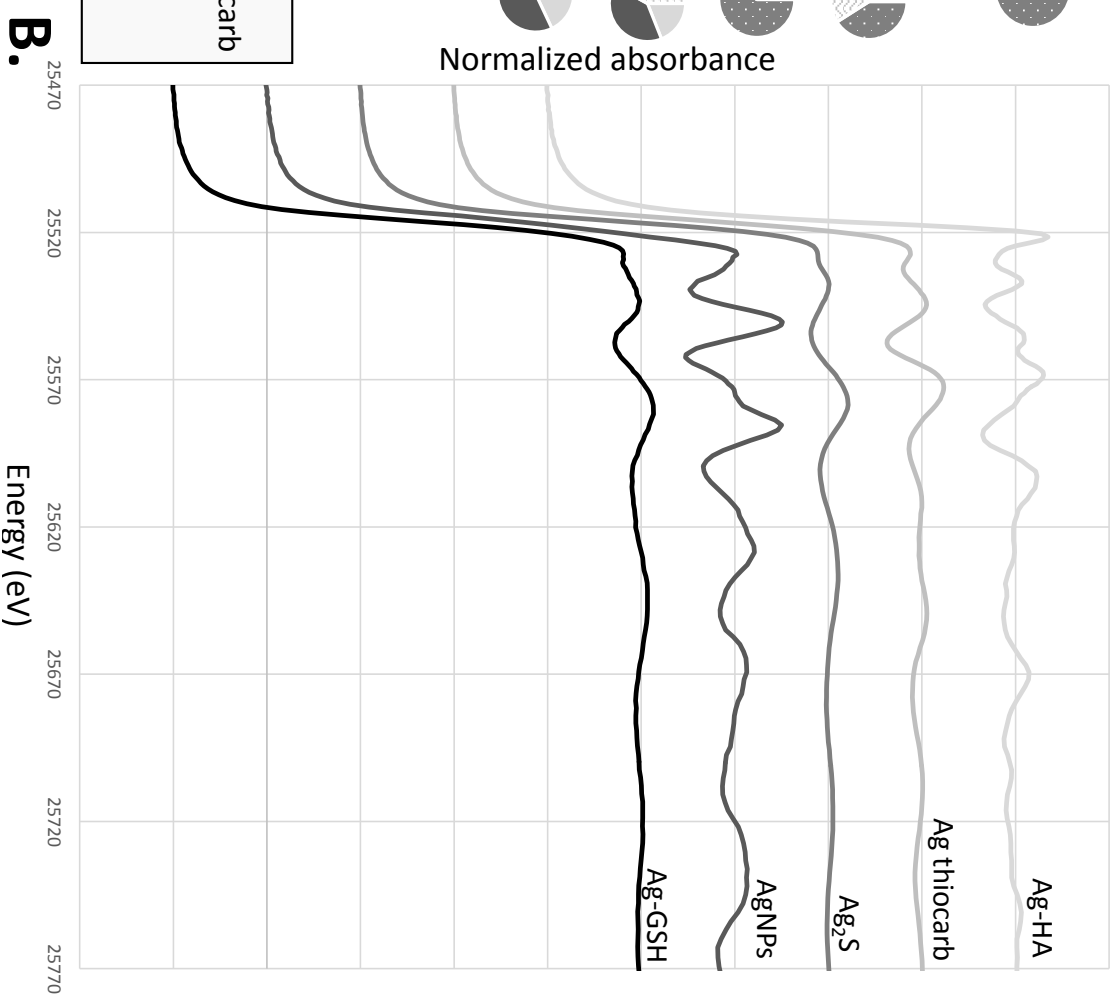


Table 1: Mean percentage of survival in the microcosms. Standard deviations are indicated.

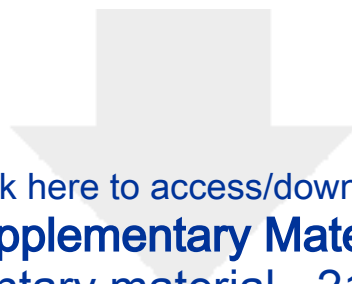
	Survival % in Perspective 3	Survival % in Perspective 6	Survival % in Perspective 10
Control	100.0 ± 0.0	97.2 ± 4.8	100.0 ± 0.0
Dis	100.0 ± 0.0	100.0 ± 0.0	97.2 ± 4.8
AgNPs	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
AgNO ₃	100.0 ± 0.0	100.0 ± 0.0	91.7 ± 14.4
AD-control	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
AD-dis	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
AD-AgNPs	100.0 ± 0.0	95.8 ± 5.9	0.0 ± 0.0
AD-AgNO ₃	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0

Table 2: Number of cocoons and juveniles in the microcosms. Standard deviations are indicated. Empty cells in the table corresponds to data no available because of the high mortality in theses conditions.

	Cocoons			Juveniles		
	Perspective 3	Perspective 6	Perspective 10	Perspective 3	Perspective 6	Perspective 10
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	0 ± 0
Dis	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	0 ± 0
AgNPs	0 ± 0	1 ± 1	0 ± 0	0 ± 0	2 ± 2	0 ± 0
AgNO ₃	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 1	0 ± 0
AD-control	13 ± 7	3 ± 1	-	5 ± 2	0	-
AD-dis	7 ± 2	18 ± 3	-	5 ± 2	2	-
AD-AgNPs	6 ± 2	10 ± 2	-	3 ± 2	1 ± 1	-
AD-AgNO ₃	32 ± 12	15 ± 9	-	5 ± 2	3 ± 1	-

Table 2: Silver concentration in mixtures (mg kg^{-1} of dry mixture). Results were obtained by ICP analysis. Standard deviations are indicated. Some standard deviations are missing due to a lack of replicates

Condition	Perspective 3	Perspective 6	Perspective 10
AD-control	2.00 ± 0.61	1.71 ± 0.23	4.57 ± 0.86
AD-AgNPs	9.54 ± 0.52	15.00 ± 1.56	23.77 ± 1.48
AD-dis	3.69 ± 0.80	2.15	8.36 ± 0.24
AD-AgNO ₃	11.63 ± 0.23	12.60	27.97 ± 5.25
AgNPs	11.23 ± 0.84	17.9 ± 1.95	27.40 ± 2.55
Dis	2.55 ± 0.35	0.45 ± 0.41	0.94 ± 0.87
AgNO ₃	13.83 ± 0.78	22.37 ± 1.76	22.37 ± 2.20
Control	0.09 ± 0.15		



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Supplementary Material

Supplementary material - 210720.docx



Legend for figure 5 in black and white (for print version) :

Figure 5: A. Speciation of silver in sewage sludge, soils and mixtures at the beginning (T0) or end (Tf) of the soil incubations and speciation of silver in earthworms: linear combination fitting of XANES spectra is shown on the left (dotted lines correspond to experimental data, and solid colored lines correspond to the fits). Samples (soils, mixtures, sewage sludge or earthworms) from conditions with AgNPs and AgNO₃ are presented. B. XANES spectra of Ag references. Ag-HA (in lightest gray) corresponds to Ag complexed with humic acids. AgNPs (in darkest gray) corresponds to the linear combination fitting obtained with a sample of NM300K AgNPs used for the experiment. Ag₂S (in dark gray) corresponds to acanthite, a silver sulfide. Ag-thiocarb (in light gray), and Ag-GSH (in black) corresponds to Ag linked to a thiol-containing organic compound.