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Abstract

The widespread use of silver nanoparticles (AgNPs) in consumer products that release Ag throughout their life cycle has raised potential environmental concerns. AgNPs primarily accumulate in soil through the spreading of sewage sludge (SS). In this study, the effects of direct exposure to AgNPs or indirect exposure via SS contaminated with AgNPs on the earthworm *Eisenia fetida* and soil microbial communities were compared, through 3 scenarios offering increasing exposure concentrations. The effects of Ag speciation were analyzed by spiking SS with AgNPs or $AgNO_3$ before application to soil. SS treatment strongly impacted Ag speciation due to the formation of Ag_2S species that remained sulfided after mixing in the soil. The life traits and expression of *lysenin*, *superoxide dismutase*, *cd-metallothionein* genes in earthworms were not impacted by Ag after 5 weeks of exposure, but direct exposure to Ag without SS led to bioaccumulation of Ag, suggesting transfer in the food chain. Ag exposure led to a decrease in potential carbon respiration only when directly added to the soil. The addition of SS had a greater effect on soil microbial diversity than the form of Ag, and the formation of Ag sulfides in SS reduced the impact of AgNPs on *E. fetida* and soil microorganisms compared with direct addition.

Keywords: Silver nanoparticles, silver sulfide, ecotoxicology, earthworms, microorganisms, speciation

1. Introduction

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

The present study sought to link silver speciation with its impact on the earthworm *Eisenia fetida*, an important soil fauna test species in ecotoxicology, and on the natural microflora. Earthworms are widely studied among soil invertebrates because they play a key role in most continental ecosystems and represent an important part of the soil macrofauna. They are considered "soil engineers" (Carbonell et al., 2009) that participate in the maintenance of soil structure and fertility. In addition to enriching the soil with organic matter available for plants, earthworms contribute to soil aeration and promote water penetration by forming galleries during their movements (Bernard et al., 2010; Carbonell et al., 2009). Several studies have shown that direct exposure to Ag in soil does not affect earthworm survival when the concentration does not exceed a few tens of mg kg^{-1} (Courtois et al., 2019). However, 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 $AgNO_3$ (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).

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

1) To assess the impact of various Ag species on earthworms' life traits (mortality, body weight gain/loss, reproduction, expression of selected genes);
2) To assess the impact of various Ag species on soil microbial diversity and soil carbon respiration activity;
3) To understand how chemical transformation of Ag in SS influences Ag toxicity compared with direct exposure.

2. Materials and methods

2.1 Earthworm test species

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

2.2. Soil

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

was collected and sieved at 5 mm. The metal content of the soil was as follows: 2.57 ± 1.97 mg 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 ± 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 mg kg^{-1}).

2.3. Sewage sludge

Four WWTPs situated in southern Poland were pre-selected in order to monitor the content of Ag during a two-year period. Among the four facilities, the facility that produced SS with the lowest level of contamination and a preferable C:N ratio was selected. According to a previous study, SS from the selected WWTP is a good source of nutrients for earthworms without inducing stress related to the presence of contaminants ((Suleiman et al., 2017)). The selected WWTP (Poland, GPS coordinates: 50°55'22.81 19°07'10.41) is a small-sized plant that uses activated sludge technology to support an agricultural area (flow: 1,000, population equivalents: 20,000). The metal content of the SS was as follows: 11.53 ± 1.43 Ag, 0.95 ± 0.22 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, 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 ± 615.99 mg kg^{-1} Zn (average based on measurements between March and December 2016). Before agricultural reuse, anaerobic stabilization of the SS was performed at laboratory scale.

2.4. Silver species

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

particles was approximately 15 nm without coating, and transmission electron microscopy (TEM) indicated a size of 17 ± 8 nm. Smaller nanoparticles of approximatively 5 nm were also present (Mendes et al., 2015). The commercial nanoparticle NM300K was kindly provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME. Each bottle contained 2 g of NM300K, which was diluted in dispersant with a volume of 2 mL; the resulting solution contained 10% (w/w) Ag (0.1 g of Ag per 1 mL). $AgNO_3$ solution was prepared by dissolving $AgNO_3$ salt in sterile distilled water. The solutions of AgNPs and Ag ions (from $AgNO_3$) were both diluted with milliQ water to obtain a Ag concentration of approximately 2 mg mL^{-1}.

2.5. Experimental scheme

2.5.1. Anaerobic digestion of sewage sludge

A batch anaerobic digestion of SS was performed in parallel in four continuous stirred-tank bioreactors. In the first bioreactor, SS was introduced without any additives (AD-control). In the second, SS was spiked with 40 mg L^{-1} NM300K AgNPs (AD-AgNPs). In the third, only a corresponding quantity of dispersant (AD-dis) was added to the SS, and in the fourth, 40 mg L^{-1} $AgNO_3$ (AD-$AgNO_3$) was added. The selected Ag concentration in the bioreactors was based on the maximum concentration of Ag that does not disturb anaerobic fermentation (Yang et al., 2012) (Full justification in SI 1)

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

- AD-$AgNO_3$: soil supplied with SS digested with $AgNO_3$ (AD-$AgNO_3$-3y, AD-$AgNO_3$-6y, AD-$AgNO_3$-10y);
- AD-control: soil supplied with SS digested without any addition (AD-control-3y, AD-control-6y, AD-control-10y).

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

- AgNPs: soil supplemented with a solution of AgNPs in dispersant (AgNPs-3y, AgNPs-6y, AgNPs-10y);
- Dis: soil supplemented with the corresponding volume of dispersant (Dis-3y, Dis-6y, Dis-10y),
- $AgNO_3$: soil supplemented with $AgNO_3$ solution ($AgNO_3$-3y, $AgNO_3$-6y, $AgNO_3$-10y)
- Control: soil without any addition.

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

2.6. Analysis

2.6.1. Biological analysis

Life traits of earthworms: reproduction, survival and biomass

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

Gene expression levels in earthworms

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

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

Microbial community composition

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

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

Potential carbon respiration

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

2.6.2. Physicochemical analysis

Metal content in earthworms and soils

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

freezing for at least 48 h and then lyophilized by group from the same microcosm for approximately 60 h. The samples were ground to a powder using liquid nitrogen and then mineralized by acid digestion (using HNO_3, H_2SO_4 and HCl_4) at high temperature as described by Bernard et al. (9). The resulting solution was analyzed by ICP-OES (inductively coupled plasma-optical emission spectrometry) (Varian 720-ES, USA) to quantify Cd, Co, Cu, Mn, Ni, Pb, Zn and Ag.

Soil samples, SS samples and mixed samples were collected and lyophilized at the beginning and end of exposure. The dried samples were ground with a mortar and pestle. For mineralization, 300 mg of sample was digested in 7 mL of concentrated HNO_3 using a Berghof microwave digestion system (speed wave MWS-2-Microwave pressure digestion). The resulting solution was analyzed by ICP-OES (Thermo apparatus) to quantify Cd, Co, Cu, Mn, Ni, Pb, Zn, and Ag.

Speciation of silver in earthworms and soils

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

Prior to analysis, the samples were lyophilized, ground and pressed into 5 mm pellets. Spectral acquisition was performed at liquid helium temperature to avoid sample evolution under the beam. Measurements were carried out in fluorescence mode using a 30-element Canberra Ge solid-state detector. Each spectrum was the sum of at least three scans. A set of model compounds including metallic Ag (AgNPs), Ag-humic acids (Ag-HA), Ag_2S, Ag-thiocarbamate (Ag-thiocarb) and Ag-glutathione (Ag-GSH) (the last two corresponding to

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

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

2.7. Bioinformatics analysis of microbial communities

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

2.8 Statistical analysis

The majority of the earthworm biomass, mortality, gene expression and metal content data did not follow a normal distribution, and the variances were not homogeneous between 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

3. Results

3.1. Biological analysis

3.1.1. Life traits: reproduction, survival and biomass

The percentage of survival of earthworms after exposure are presented in Table 1. Statistical tests showed no significant differences between any of the treatments and their respective controls without Ag (i.e. the control of AgNPs is Dis, for $AgNO_3$ it is Control, for AD-AgNPs it is AD-dis and for AD-$AgNO_3$ it is AD-control). Ag did not cause mortality under these conditions. A significant impact of SS addition was observed in treatment AD-X-10y (perspective 10) with or without Ag. The addition of the highest dose of digested SS led to the death of all earthworms living in the microcosms.

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

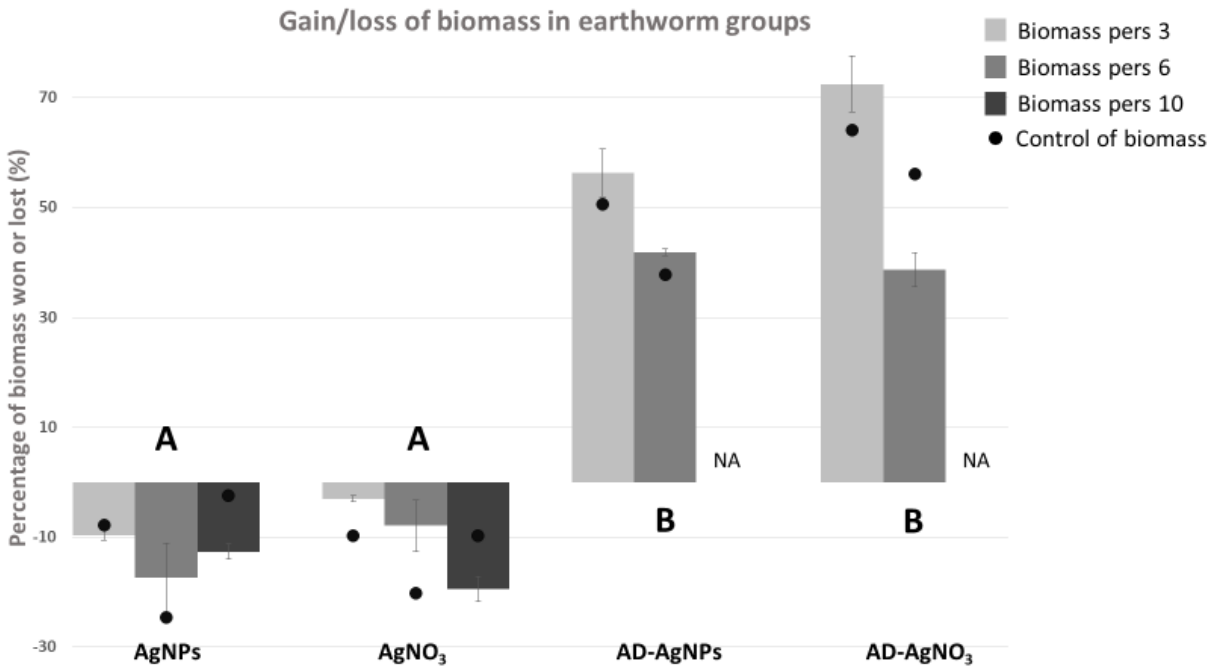
	Survival % in Perspective 3	Survival % in Perspective 6	Survival % in Perspective 10
Control	100.0 ± 0.0	97.2 ± 4.8	100.0 ± 0.0
Dis	100.0 ± 0.0	100.0 ± 0.0	97.2 ± 4.8
AgNPs	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
$AgNO_3$	100.0 ± 0.0	100.0 ± 0.0	91.7 ± 14.4
AD-control	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
AD-dis	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

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

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	-

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



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 $AgNO_3$, 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, $AgNO_3$, AD-AgNPs and AD-$AgNO_3$ (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.

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

3.1.3 Potential carbon respiration

All microorganism activity and diversity measurements were performed only for perspective 3. After direct exposure to Ag (AgNPs or $AgNO_3$) in soil, a strong and significant decrease in potential carbon respiration was observed compared to the control and dispersant-treated soils. There was no effect of Ag form initially added in soil because the impact of AgNPs and $AgNO_3$ was similar on the potential respiration. No effect on potential carbon respiration was observed when Ag (AgNPs or $AgNO_3$) was applied on soil via SS (Figure 2), with a positive effect of SS application on microbial carbon respiration.

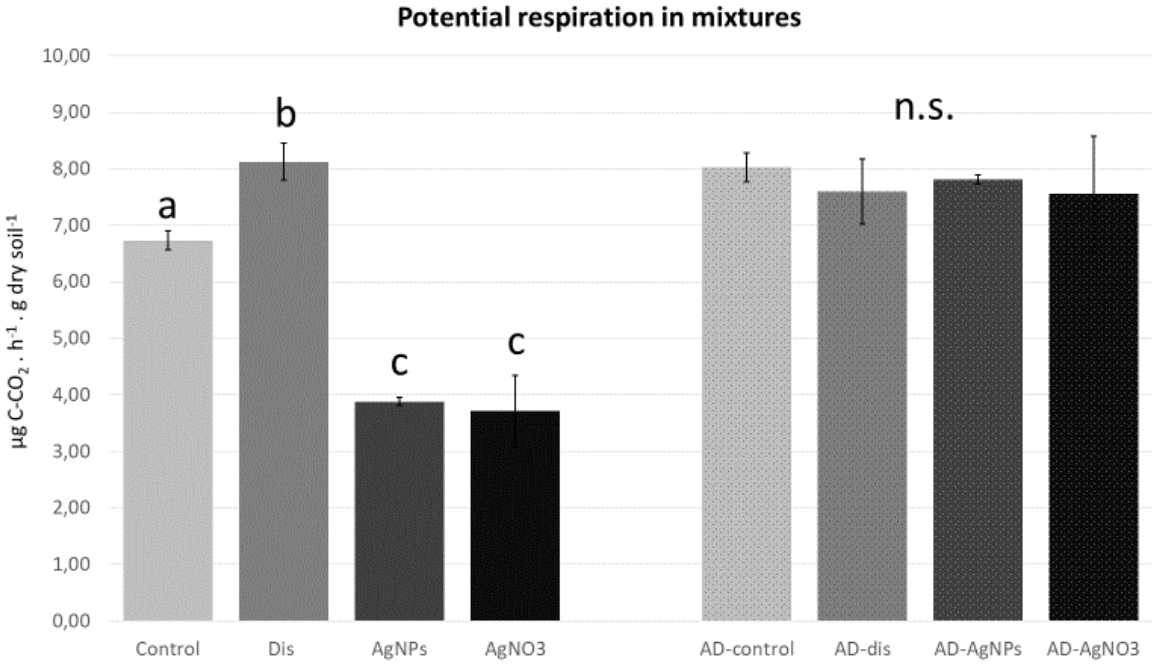


Figure 2: Potential carbon respiration of microbial communities in mixtures (µg C-CO_2.h^{-1}.g^{-1} of dry soil). The letters indicate significant differences in respiration between the 4 treatments without sewage sludge. "n.s." indicates that there were no significant differences between the 4 treatments with sewage sludge.

3.1.4. Microbial community composition

After 5 weeks of the experiment, microbial community composition differed greatly depending on the presence or absence of SS. The addition of SS explained 52% of the variance between these 2 groups (Figure 3). Ag addition (with or without SS) explained another 12% of the variance. Interactions between routes of entry (direct exposure or via SS application) and Ag form (AgNPs or $AgNO_3$) explained 12% of the variance.

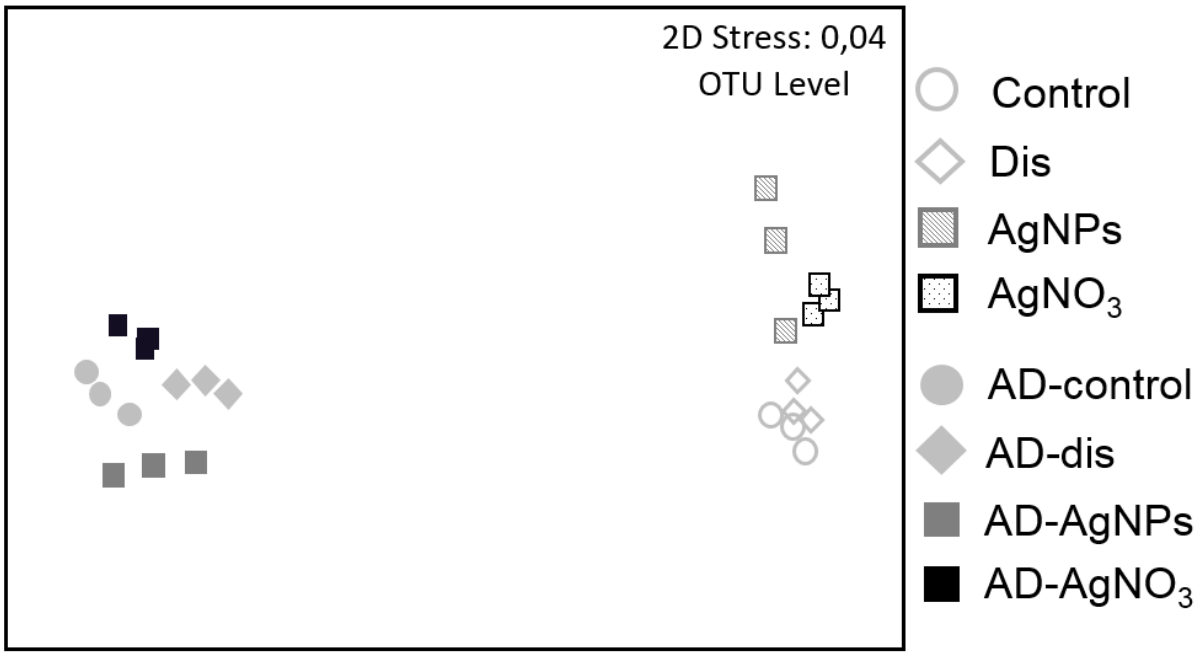


Figure 3: Two-dimensional ordination solution using non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance showing the relative proximities of microbial community composition among all treatments.

The IndVal analysis (based on 519 OTU) failed to extract any indicator of Ag exposure at the species or genus level (Table SI 8). However, the Indval analysis based on samples supplied with or without SS led to identify 118 microorganisms indicating of SS supply. Among them, we found 9 Archaea from the *Methanobacteriales* and *Methanomicrobiales* orders and 109 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 ± 1.69 mg kg^{-1}, 16.97 ± 3.99 mg kg^{-1} and
456 25.38 ± 3.69 mg kg^{-1}, respectively. In perspectives 3 and 10, the Ag concentrations in AD-
457 AgNPs, AD-$AgNO_3$, AgNPs and $AgNO_3$ 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

The contents of 7 metals in earthworms were analyzed after exposure (Table SI 9). Since the organisms exposed to SS in perspective 10 did not survive, metal content data were not available for these microcosms. No significant variation in Pb and Zn was observed regardless of the treatment and perspective. Cd, Cu, and Mn were bioaccumulated differently by the earthworms depending on exposure to SS, with greater bioaccumulation in the presence of SS. However, there was no impact of Ag on the bioaccumulation of these metals. The concentrations of Cu and Mn in worms increased with their concentrations in the soil (presence or absence of SS in the microcosms). Conversely, the bioaccumulation of Cd was lower in the presence of higher concentrations of Cd in the soil.

In all treatments without Ag addition, the Ag content in earthworms was below the detection level. Bioaccumulation of Ag in earthworms was significant when they were exposed to microcosms directly contaminated with Ag (Figure 4). In microcosms with SS application, Ag bioaccumulation in earthworms was significant only in the condition AD-$AgNO_3$-6y and was detectable in AD-AgNPs-3y, AD-AgNPs-6y and AD-$AgNO_3$-3y.

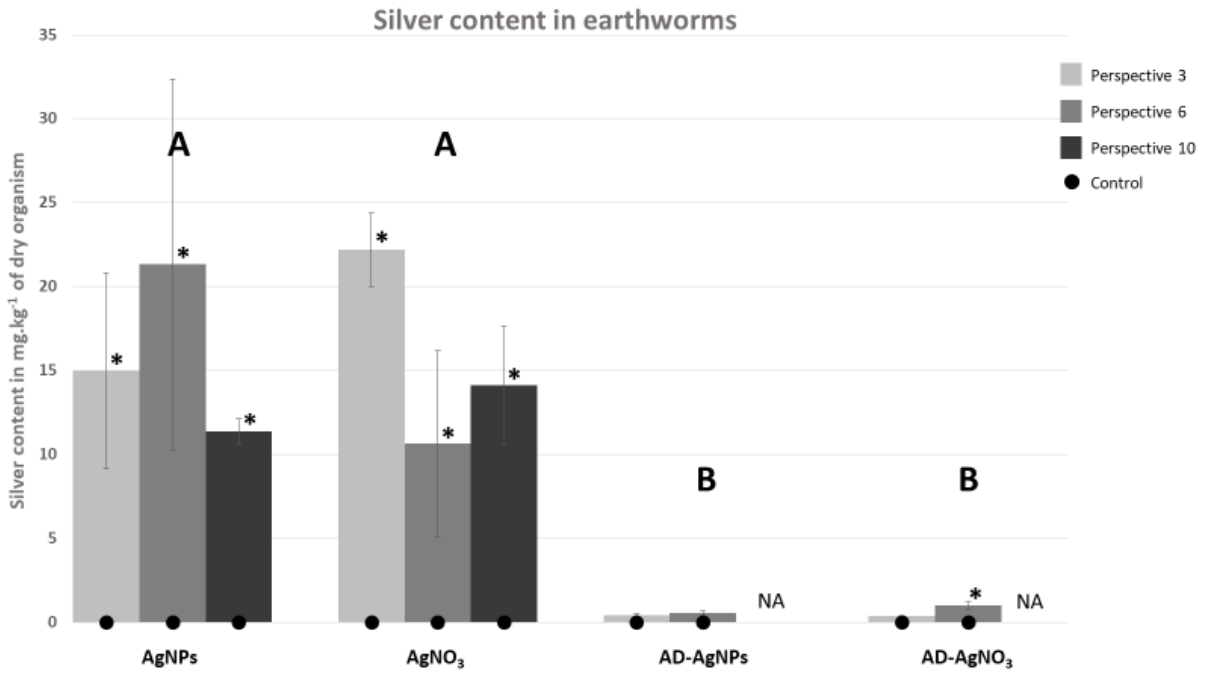


Figure 4: Silver content in earthworms (mg of Ag kg^{-1} of dry matter). Each perspective is represented by a color: light grey for perspective 3, medium grey for perspective 6 and dark grey for perspective 10. NA means data are non-available due to the high mortality in these conditions. The big letters indicate significant differences among conditions AgNPs, $AgNO_3$, AD-AgNPs and AD-$AgNO_3$ (no difference between perspectives within the same condition). The black dots correspond to the mean biomass of earthworms in the control (dispersant for AgNPs and control for $AgNO_3$). The stars (*) indicate significantly different values compared with the associated control without silver.

3.2.3. Speciation of silver in soil

AgNPs and $AgNO_3$ in soil

In the absence of SS, initially (approximately 2 h after spiking), the speciation of Ag varied depending on its initial form (Figure 5). Ag initially from AgNPs was mainly in its pristine form (76% metallic Ag), with the remainder bound to organic matter (24%). This latter fraction was probably the fraction of soluble Ag that quickly dissolved and was complexed by the organic matter present in the soil. The Ag initially from $AgNO_3$ was quickly complexed with organic matter with a fraction of thiol groups (21% Ag-thiol). Importantly, after the 5 weeks of the experiment, Ag speciation no longer depended on the initial speciation. In the presence of either AgNPs or $AgNO_3$, approximately 40% of Ag was in the metallic form, 40% was complexed to an organic thiol, and 20% was bound to humic acids.

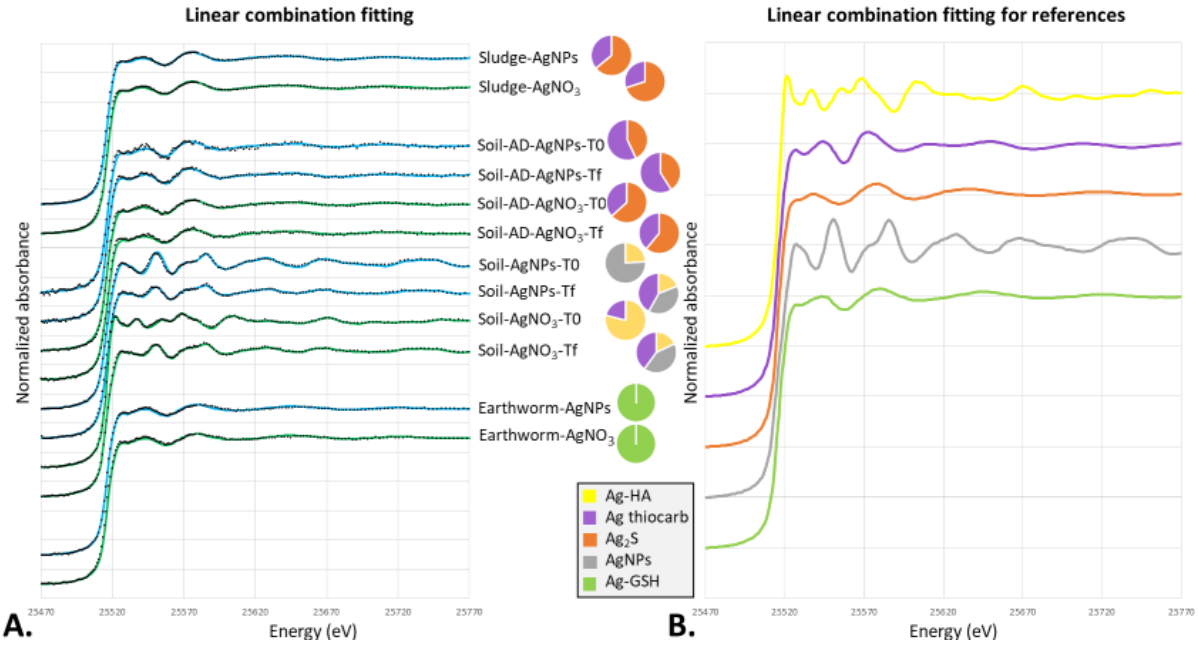


Figure 5: A. Speciation of silver in sewage sludge, soils and mixtures at the beginning (T0) or end (Tf) of the soil incubations and speciation of silver in earthworms: linear combination fitting of XANES spectra is shown on the left (dotted lines correspond to experimental data, and solid colored lines correspond to the fits). Samples (soils, mixtures, sewage sludge or earthworms) from conditions with AgNPs are in blue. Samples from conditions with $AgNO_3$ are in dark green. B. XANES spectra of Ag references. Ag-HA (in yellow) corresponds to Ag complexed with humic acids. AgNPs (in gray) corresponds to the linear combination fitting obtained with a sample of NM300K AgNPs used for the experiment. Ag_2S (in orange) corresponds to acanthite, a silver sulfide. Ag-thiocarb (in purple), and Ag-GSH (in light green) corresponds to Ag linked to a thiol-containing organic compound.

AD-AgNPs and AD-$AgNO_3$ in soil

When spiked in SS prior to addition to soil, the Ag in the SS was transformed into Ag_2S (30-33%) and Ag bound to a thiol-containing organic compound (67-70%), regardless of the initial state of the Ag (ionic or NPs) (Figure 5). These two species remained the main species after the addition of SS to the soil, with small variations in the relative proportions of the two species. The speciation of Ag did not change after the 5 weeks of the experiment.

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-$AgNO_3$ 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 $AgNO_3$. 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 $AgNO_3$) led to
542 significant bioaccumulation of Ag in earthworms (10–20 mg kg^{-1}), whereas the addition of the

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

Regardless of its form and concentration, the presence of Ag did not alter the expression of lysenin (*lys*), superoxide dismutase (*sod*) and cadmium metallothionein (*cdmt*) genes in earthworms after 5 weeks of exposure. These genes, which are involved in oxidative stress (Choi and Park, 2015), immunity (Hayashi et al., 2015) and defense against toxic metal ions (Hayashi et al., 2013), have been studied previously in the context of Ag. According to the literature, AgNPs and $AgNO_3$ at high concentration (500 mg kg^{-1}) in natural soil does not affect the expression of the *sod* gene (Hayashi et al., 2013) while in artificial soil, an overexpression of sod has been observed with 100 mg kg^{-1} of $AgNO_3$ only (Choi and Park, 2015). In this last cited study, AgNPs did not affect the *sod* gene expression, even at low concentration (1 to 100 mg kg^{-1}). Only one study showed the effect of Ag on the lys gene expression with in-vitro conditions. Authors exposed coelomocytes of earthworms to solutions of AgNPs and $AgNO_3$ at low concentrations during 24 hours and saw a rapid upregulation (measure after 2 h of exposure) of *lys* only this AgNPs and a late down-regulation (measure after 8 or 24 h of exposure) (Hayashi et al., 2015). *Cdmt* gene has also been studied a little in the context of 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) (Demuynck 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.

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

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

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

Thus, the lasting effects of introduction of Ag to the terrestrial environment via SS were slight changes in the soil microbial community and slight but not necessarily significant accumulation of Ag in earthworms. There was no effect of the form of Ag initially provided or of the Ag concentration in the microcosms. Thus, the effects of Ag in SS were strongly attenuated compared with direct introduction of Ag (2 forms) into the soil. These observations can be explained by Ag speciation in soil. During the anaerobic fermentation of SS, Ag underwent chemical transformations. Both $AgNO_3$ and AgNPs changed in the same manner, and at the end of fermentation, the two forms of Ag were completely sulfided, consistent with previous studies (Levard et al., 2012; Pradas del Real et al., 2016). After mixing in the soil, the Ag remained completely sulfided. These chemical forms were stable and did not evolve much during the 5 weeks of incubation. Silver sulfidation strongly decreases silver toxicity to a variety of (micro-)organisms due to the high chemical stability of the Ag-S species (Levard et al., 2013, 2011; Reinsch et al., 2012). Likewise, when introduced directly into soil, the speciation of $AgNO_3$ and AgNPs evolved in a similar manner by the end of 5 weeks (with not more than 40% of Ag bound to sulfur molecules). Thus, in both modes of supply, AgNPs and

643 $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_3$ 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

670 The authors declare no competing financial interest.

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681

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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 multidisciplinarity 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).

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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,

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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 (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

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:

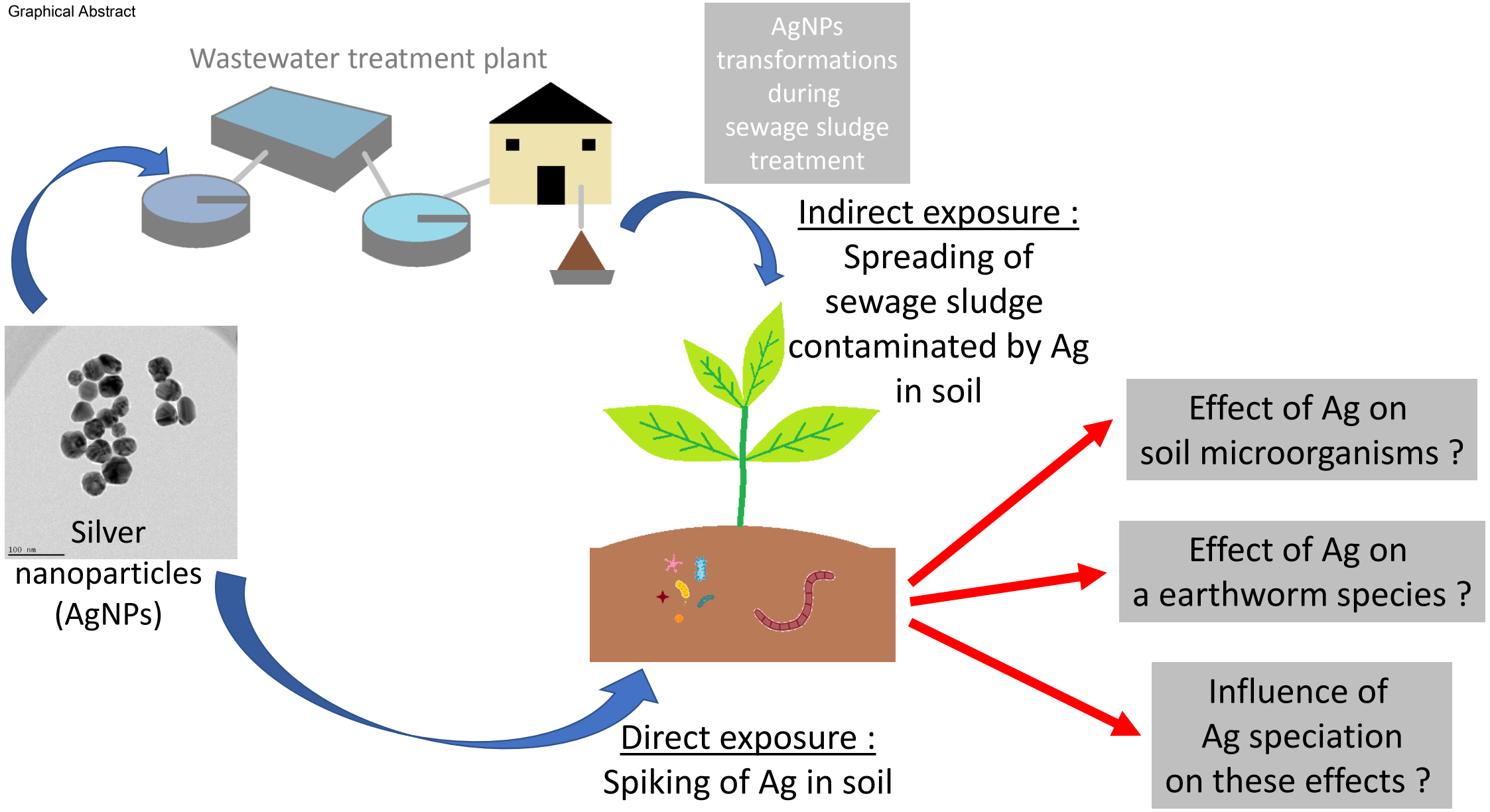
- Ag is brought to the agricultural soil from spreading of contaminated sewage sludge
- *Eisenia fetida* bioaccumulate few amount of Ag in realistic scenario of exposure
- In realistic scenario, Ag change a little the diversity of soil microbial communities
- Speciation of Ag in sewage sludge causes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

Graphical Abstract



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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Abstract

The widespread use of silver nanoparticles (AgNPs) in consumer products that release Ag throughout their life cycle has raised potential environmental concerns. AgNPs primarily accumulate in soil through the spreading of sewage sludge (SS). In this study, the effects of direct exposure to AgNPs or indirect exposure via SS contaminated with AgNPs on the earthworm *Eisenia fetida* and soil microbial communities were compared, through 3 scenarios offering increasing exposure concentrations. The effects of Ag speciation were analyzed by spiking SS with AgNPs or $AgNO_3$ before application to soil. SS treatment strongly impacted Ag speciation due to the formation of Ag_2S species that remained sulfided after mixing in the soil. The life traits and expression of *lysenin*, *superoxide dismutase*, *cd-metallothionein* genes in earthworms were not impacted by Ag after 5 weeks of exposure, but direct exposure to Ag without SS led to bioaccumulation of Ag, suggesting transfer in the food chain. Ag exposure led to a decrease in potential carbon respiration only when directly added to the soil. The addition of SS had a greater effect on soil microbial diversity than the form of Ag, and the formation of Ag sulfides in SS reduced the impact of AgNPs on *E. fetida* and soil microorganisms compared with direct addition.

Keywords: Silver nanoparticles, silver sulfide, ecotoxicology, earthworms, microorganisms, speciation

Figure 1

Click here to access/download;Figure;Fig 1 biomass.pptx

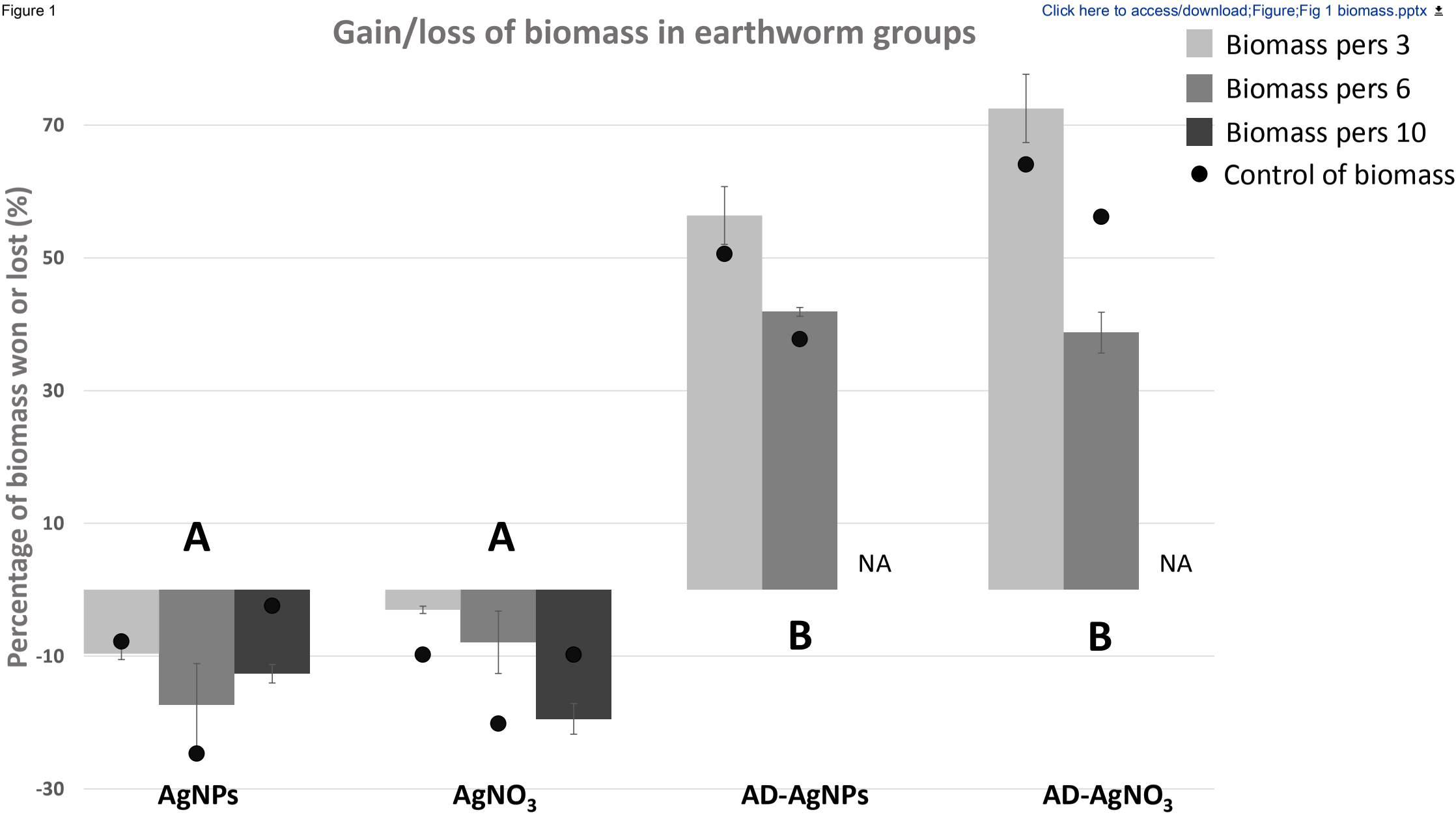

Figure 2

Click here to access/download;Figure;Fig 2 respiration sols.pptx

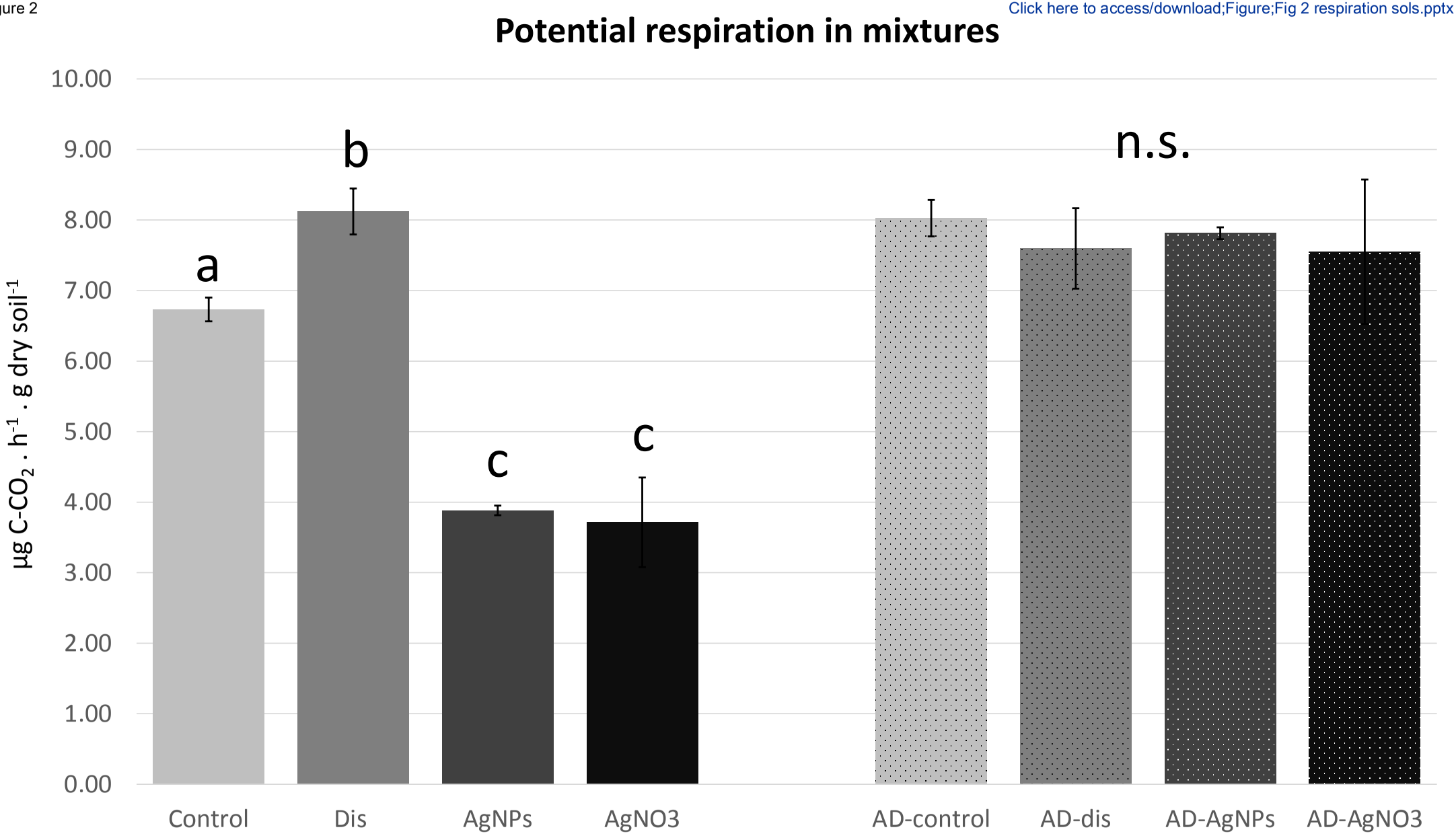

Figure 3

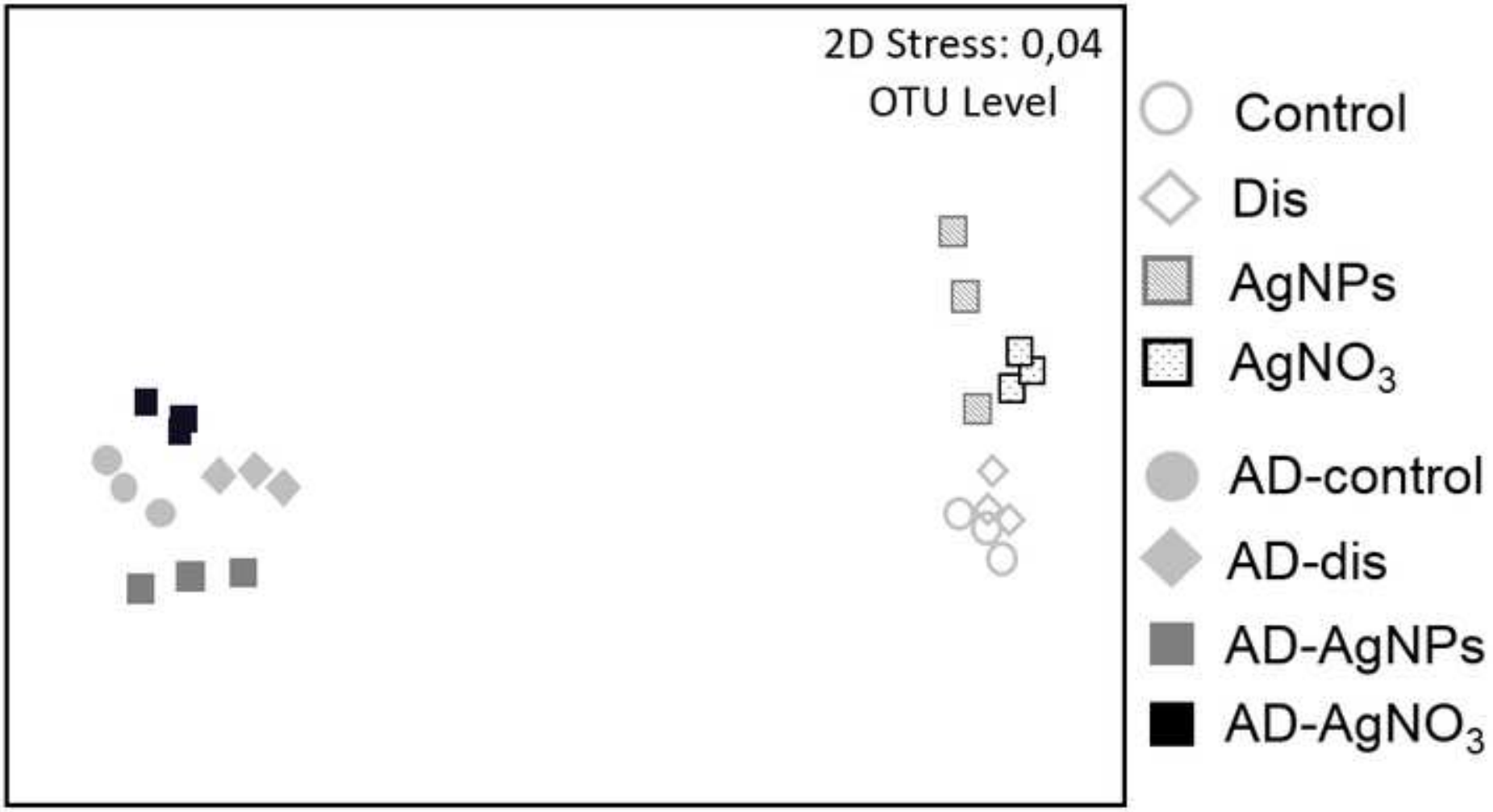

Figure 4

Click here to access/download;Figure;Fig 4 Ag in earthworms.pptx

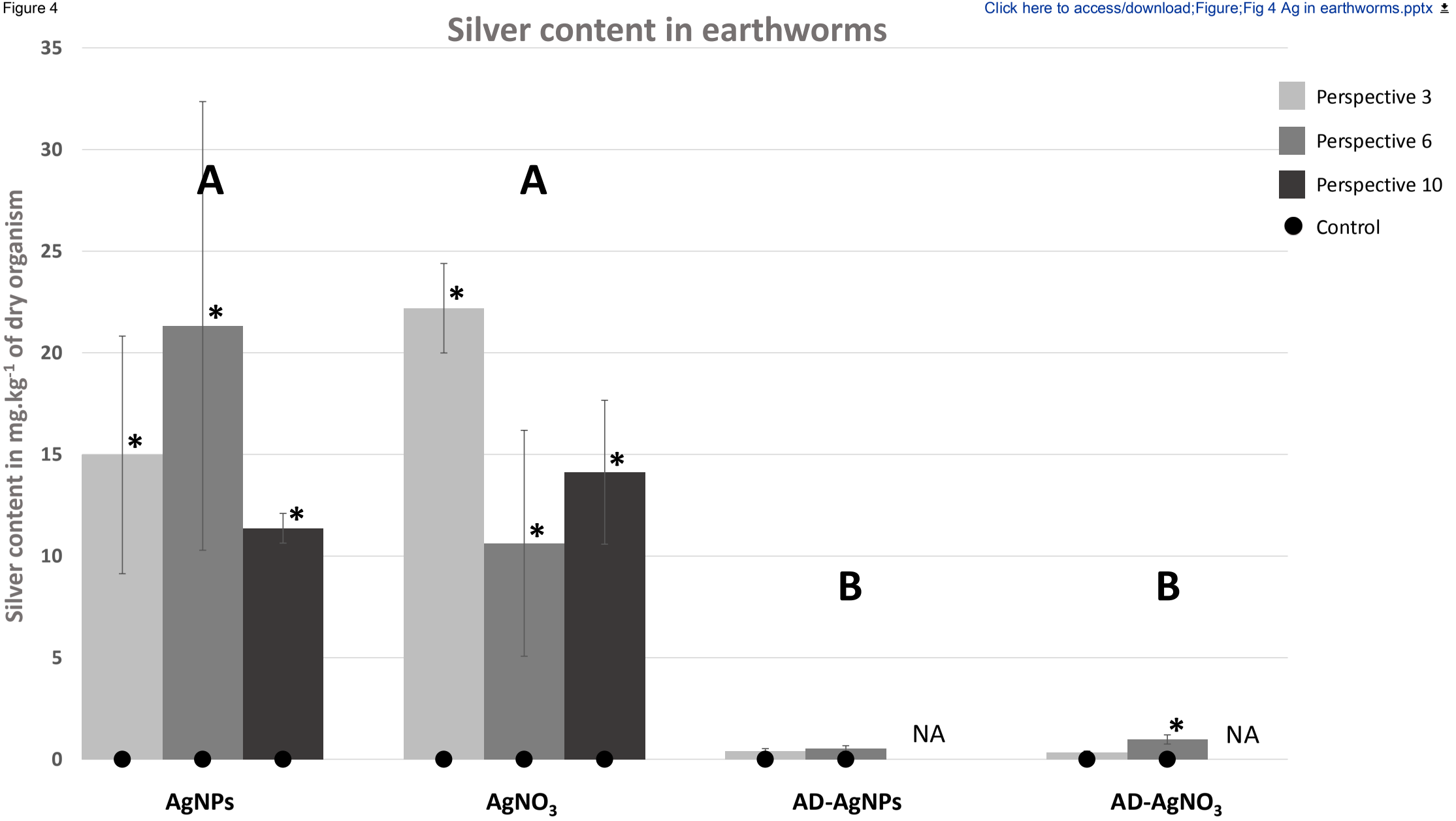

Figure 5 (color)

Click here to access/download;Figure;Fig 5 speciation (color).pptx

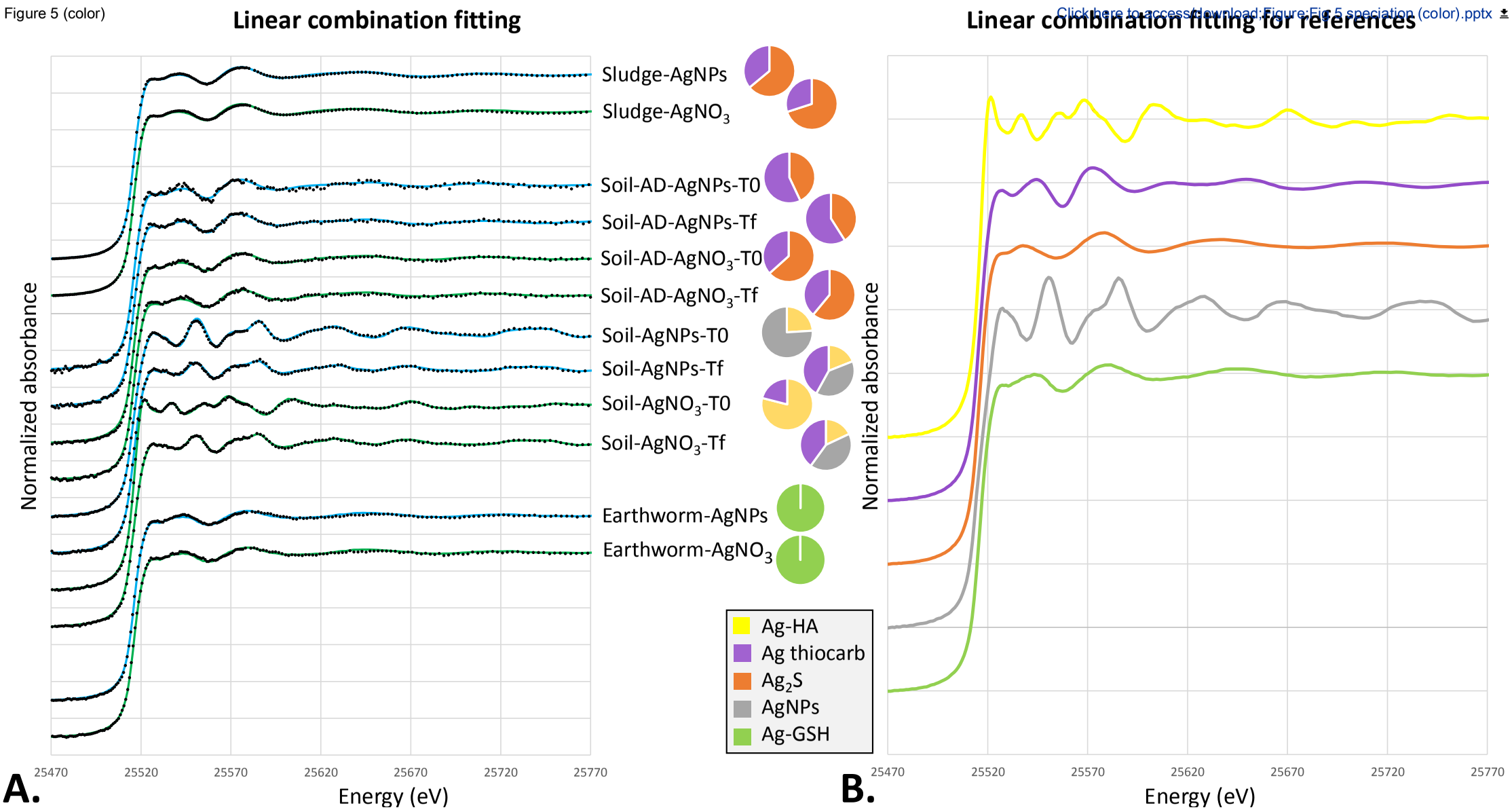

Figure 5 (colorless)

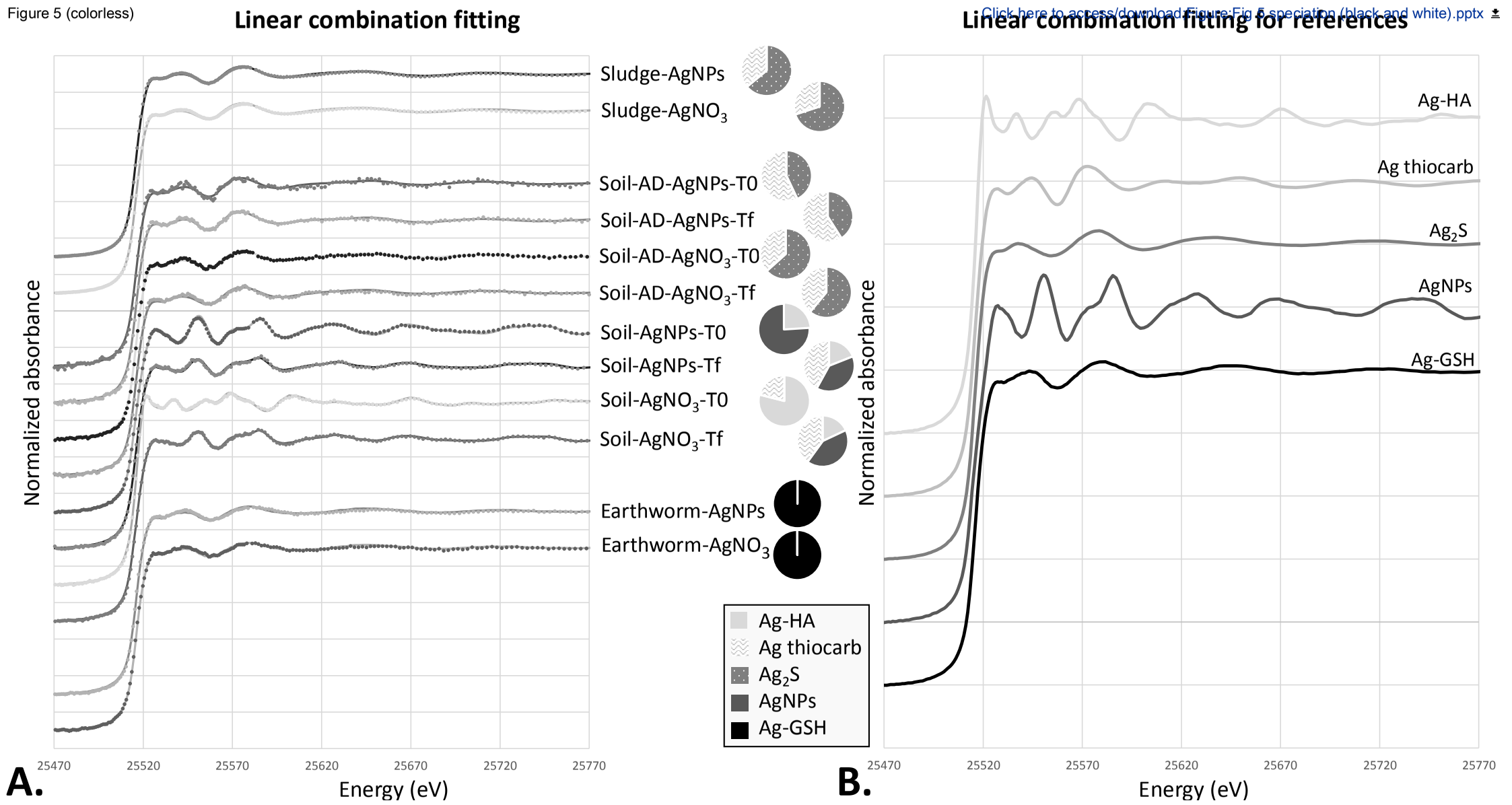

Table 1: Mean percentage of survival in the microcosms. Standard deviations are indicated.

	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		

Supplementary Material

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 $AgNO_3$ 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. Ag_2S (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.