# THÈSE de DOCTORAT

Préparée par **Pauline COURTOIS**

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Discipline : Géosciences, Ecologie, Paléontologie, Océanographie

Spécialité : Biologie de l'environnement, Ecotoxicologie

# Evaluation écotoxicologique des nanoparticules d'argent et leurs dérivés : leurs effets sur la faune, la flore et les microorganismes du sol.

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# Ecotoxicological assessment of silver nanoparticles and their derivatives: their effects on fauna, flora and soil microorganisms.

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Directeur de thèse : **Pr. Franck VANDENBULCKE**

Université de Lille

Ecole doctorale Science de la Matière, du Rayonnement et de l'Environnement

Laboratoire de Génie Civil et géo-Environnement

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| | | |
|---|---|---|
| Mme Camille DUMAT | Professeure, Univ. de Toulouse, INP-ENSAT | Rapporteuse |
| Mme Maria Soledad GONI URRIZA | Maître de conférence, Univ. de Pau et des Pays de l'Adour, IPREM | Rapporteuse |
| Mme Juliette FABURE | Maître de conférence, Univ. Paris-saclay, AgroParisTech | Examinatrice |
| M. Jérome ROSE | Directeur de recherche, Univ. Aix Marseille, CNRS CEREGE | Examinateur |
| M. Damien CUNY | Professeur, Univ. de Lille, Faculté de pharmacie, LGCgE | Président du jury |
| M. Franck VANDENBULCKE | Professeur, Univ. de Lille, Campus scientifique, LGCgE | Directeur de thèse |

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## RÉSUMÉ

De plus en plus de produits manufacturés contiennent des nanoparticules d'argent (AgNPs) qui sont, entre autres, incorporées pour leur excellente propriété biocide. Le cycle de vie de ces produits nanofonctionnalisés génère des rejets considérables dans l'environnement, notamment dans les eaux usées. L'efficacité des stations d'épuration permet de piéger dans les boues la plupart des espèces d'argent (Ag). Cependant, les boues d'épuration sont souvent recyclées par épandage sur des terres agricoles. Le sol est donc le principal lieu de dépôt des contaminants des boues d'épuration. En raison de la quantité de boues produite et épandue chaque année, il est devenu important d'évaluer avec précision l'impact des espèces d'Ag telles qu'elles sont introduites dans l'environnement.

Ce travail de thèse a consisté en une évaluation globale de l'effet, sur l'écosystème terrestre, des formes d'Ag apportées par les boues dans le sol. Pour cela, une analyse de spéciation de l'Ag, apporté dans les sols par des boues d'épuration digérées, a été réalisée. Divers organismes animaux, végétaux et microbiens ont été exposés à cette forme d'Ag, de manière plus ou moins réaliste selon les besoins de l'étude, afin d'en évaluer les différents effets néfastes.

Les résultats indiquent que les AgNPs se retrouvent complètement sulfurées suite à une digestion anaérobie des boues d'épuration. Sous cette forme chimique, l'Ag est plus faiblement toxique que les AgNPs, pour tous les organismes animaux, végétaux et microbiens étudiés. L'Ag sulfuré est également plus faiblement biodisponible pour ces organismes, bien qu'il puisse être légèrement bioaccumulé par les animaux et végétaux et ainsi entrer tout de même dans les chaînes trophiques. Néanmoins, un effet durable sur les communautés microbiennes a été observé. Cet effet diffère selon la nature du sol recevant les boues, et semble perturber le cycle des nutriments azotés.

## MOTS CLÉS

Écotoxicologie, nanoparticules d'argent, sulfure d'argent, spéciation, écosystème terrestre, sols, macrofaune du sol, flore terrestre, microorganismes du sol

## ABSTRACT

More and more manufactured products contain silver nanoparticles (AgNPs) which, among other things, are incorporated for their excellent biocidal property. The life cycle of these nanofunctionalized products generates considerable releases into the environment, particularly in wastewater. The efficiency of wastewater treatment plants makes it possible to trap most species of silver (Ag) in the sludge. However, sewage sludge is often recycled by land application on agricultural land. Soil is the main place of deposition of contaminants in sewage sludge. Due to the amount of sewage sludge produced and spread each year, it has become important to accurately assess the impact of Ag species as they are introduced into the environment.

This thesis work consisted of a global evaluation of the effect, on the terrestrial ecosystem, of the forms of Ag brought by the sewage sludge in the soil. For this, an analysis of speciation of Ag brought in soils via digested sewage sludge was carried out. Various animal, plant and microbial organisms have been exposed to this form of Ag, more or less reastically depending on the needs of the study, in order to assess its various harmful effects.

The results indicate that AgNPs become completely sulfided following anaerobic digestion of sewage sludge. In this chemical form, Ag is less toxic than AgNPs, to all animals, plants and microbial organisms studied. Ag sulfide is also less bioavailable to these organisms, although it may be slightly bioaccumulated by animals and plants and thus enters food chains. Likewise, an effect may persist on microbial communities. This effect differs depending on the type of soil receiving the sewage sludge, and seems to disrupt the nitrogen nutrient cycle.

## KEYWORDS

Ecotoxicology, silver nanoparticles, silver sulfide, speciation, terrestrial ecosystem, soils, soil macrofauna, terrestrial plants, soil microorganisms

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Pauline Courtois, Anna Grosser, Agnieszka Rorat, Sébastien Lemiere, Christine Lors, Anna Grobelak, Franck Vandenbulcke, 2021?. Impact of silver forms brought by sewage sludge spreading on two different soils on the test species *Eisenia fetida* in realistic conditions. *(Sera soumis prochainement)*

Pauline Courtois, Annette de Vaufleury, Anna Grosser, Christine Lors, Franck Vandenbulcke, 2021. Transfer of sulfidized silver from silver nanoparticles, in sewage sludge, to plants and primary consumers in agricultural soil environment. Science of the Total Environment, in press. https://doi.org/10.1016/j.scitotenv.2021.145900

Pauline Courtois, Agnieszka Rorat, Sébastien Lemiere, Rémy Guyoneaud, Eléonore Attard, Manon Longepierre, François Rigal, Clément Levard, Perrine Chaurand, Anna Grosser, Anna Grobelak, Malgorzata Kacprzak, Christine Lors, Agnès Richaume, Franck Vandenbulcke, 2020. Medium-term effects of Ag supplied directly or via sewage sludge to an agricultural soil on *Eisenia fetida* earthworm and soil microbial communities. Chemosphere, 128761. https://doi.org/10.1016/j.chemosphere.2020.128761

Pauline Courtois, Agnieszka Rorat, Sébastien Lemiere, Clément Levard, Perrine Chaurand, Anna Grobelak, Christine Lors, Franck Vandenbulcke, 2020. Accumulation, speciation and localization of silver nanoparticles in the earthworm *Eisenia fetida*. Environmental Science and Pollution Research p. 10. https://doi.org/10.1007/s11356-020-08548-z

Pauline Courtois, Agnieszka Rorat, Sébastien Lemiere, Rémy Guyoneaud, Eléonore Attard, Clément Levard, Franck Vandenbulcke, 2019. Ecotoxicology of silver nanoparticles and their derivatives introduced in soil with or without sewage sludge: A review of effects on microorganisms, plants and animals. Environmental Pollution 253, 578–598. https://doi.org/10.1016/j.envpol.2019.07.053

Agnieszka Rorat, Pauline Courtois, Franck Vandenbulcke, Sébastien Lemiere, 2019. Sanitary and environmental aspects of sewage sludge management in Prasad, M.N.V., de Campos Favas, P.J., Vithanage, M., Mohan, S.V. (Eds.), Industrial and Municipal Sludge. Butterworth-Heinemann, pp. 155-180. https://doi.org/10.1016/b978-0-12-815907-1.00008-8

## Communications orales

- "Journée des doctorants" de l'IRePSE (Octobre 2019, France, Villeneuve d'Ascq) - Effects of silver forms contained in sewage sludge in *Eisenia fetida* earthworms.

- SETAC (Society of Environmental Toxicology and Chemistry) (Mai 2019, Finlande, Helsinki) – Ecotoxicology of silver species brought by sewage sludge in terrestrial environment.

## Autres communications (posters)

- YES (Young Environmental Scientists) Meeting (Février 2019, Belgique, Gand) - Ecotoxicology of silver species brought by sewage sludge in terrestrial environment.

- "Journée de la recherche" du BDE PolySciences Lille (Février 2019, France, Villeneuve d'Ascq).

- International Conference of Environmental Biotechnology (Décembre 2017, Pologne, Czestochowa) - Sewage sludge, silver nanoparticles and terrestrial environments: state of the art and involved work.

- SEFA (Société d'Ecotoxicologie Fondamentale et Appliquée) (Juin 2017, France, Lille) - Boues de STEP, nanoparticules d'argent et environnements terrestres : état des lieux et travaux engagés.

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# LISTE DES ABREVIATIONS

| | |
|---|---|
| **µA** | microampère |
| **Ag** | Argent |
| **$Ag^+$** | Ion argent |
| **AgCl** | Chlorure d'argent |
| **Ag-HA** | Argent lié à des acides humiques |
| **AgMPs** | Microparticules d'argent |
| **AgMNPs** | Microparticules et nanoparticules d'argent |
| **$AgNO_3$** | Nitrate d'argent |
| **AgNPs** | Nanoparticules d'argent |
| **$Ag_2S$** | Sulfure d'argent |
| **$Ag_2SO_4$** | Sulfate d'argent |
| **$Ag(S_2O_3)^-$** | Thiosulfate d'argent |
| **Ag-thio** | Argent lié à un thiocarbamate |
| **Al** | Aluminium |
| **ANSES** | Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail |
| **AOB** | Bactéries oxidant l'ammonium |
| **As** | Arsenic |
| **Au** | Or |
| **B** | Bore |
| **BSA** | Albumine de sérum bovin (revêtement) |
| **C** | Carbone |
| **Ca** | Calcium |
| **CAT** | Catalase (protéine) |
| ***cat*** | Gène codant pour la catalase |
| **Cd** | Cadmium |
| **CdMT** | Cadmium-métallothionéine (protéine) |
| ***cdmt*** | Gène codant pour la Cadmium-métallothionéine |
| **$CO_2$** | Dioxyde de carbone |
| **Cr** | Chrome |

| | |
|---|---|
| **CTAB** | Bromure de cétrimonium (revêtement) |
| **Cu** | Cuivre |
| **3D** | 3 dimensions |
| **d** | Days - jours |
| **DAF-12** | Gène codant pour la protéine « abnormal dauer formation protein 12 » (récepteur nucléaire de l'acide dafachronique) chez *C. elegans* |
| **Dis** | Solution dispersante |
| **d.m.** | Dry matter |
| **DNA** | ADN Acide Désoxyribonucléique |
| **EC30** | 30% effective concentration – concentration à laquelle on observe un effet chez 30% des organismes tests |
| **FOV** | Field of view – champ de vue |
| **GA** | Gomme arabique (revêtement) |
| **GSH** | Glutathion |
| **GSV** | Gray Scale Value – valeur d'échelle de gris |
| **h** | Hours - heures |
| **HA** | Humic acids – acides humiques |
| **$HClO_4$** | Acide perchlorique |
| **$HNO_3$** | Acide nitrique |
| **$H_2SO_4$** | Acide sulfurique |
| **ICP-OES** | Inductively coupled plasma - optical emission spectrometry - Spectroscopie d'émission atomique à plasma à couplage inductif |
| **KCl** | Chlorure de potassium |
| **kg** | Kilogramme |
| **kV** | Kilovolt (tension) |
| **LCF** | Linear combination fitting – ajustement par combinaison linéaire |
| **LUFA** | Sols standardisés vendus par LUFA Speyer |
| **LYS** | Lysénine (protéine) |
| ***lys*** | Gène codant pour la lysénine |
| **MAPK** | Mitogen-activated protein kinase – Protéines kinases participant à l'induction de la mitose |
| **mg** | Milligramme |

| | |
|---|---|
| **Mg** | Magnésium |
| **microX-CT** | X-ray micro-computed tomography - microtomographie aux rayons X |
| **mL** | Millilitre |
| **mm** | Millimètre |
| **$mm^3$** | Millimètre cube (volume) |
| **Mn** | Manganèse |
| **$MnO_2$** | Dioxyde de manganèse |
| **Mo** | Molybdène |
| **mPEG** | Methoxypolyethylene glycol (revêtement) |
| **MPs** | Microparticules |
| **m.s.** | Matière sèche |
| **MT ou MTs** | Métallothionéine(s) (protéines) |
| ***mt*** | Gène codant pour les métallothionéines |
| **Ni** | Nickel |
| **nm** | Nanomètre |
| **NM** | Nanomatériaux |
| **$N_2O$** | Protoxyde d'azote |
| **NPs** | Nanoparticules |
| **OA** | Acide oléique |
| **OECD** | OCDE Organisation de coopération et de développement économiques |
| **OTU** | Operational Taxonomic Unit - Unité taxonomique opérationnelle |
| **Pb** | Plomb |
| **pH** | Potentiel hydrogène |
| **ppm** | Partie par million |
| **PVA** | Alcool polyvinylique (revêtement) |
| **PVP** | Polyvinylpyrrolidone (revêtement) |
| **ROS** | Espèce réactive de l'oxygène |
| **s** | Seconds - secondes |
| **sAgNPs** | Nanoparticules d'argent sulfurées |
| **SOD** | Superoxyde dismutase (protéine) |

| | |
|---|---|
| ***sod*** | Gène codant pour la superoxyde dismutase |
| **SS** | Sewage sludge - Boue d'épuration |
| **STEP** | Station d'épuration |
| **T0** | Initial time – Début de l'expérience |
| **TA** | Acide tannique (revêtement) |
| **Tf** | Final time – Fin de l'expérience |
| **w** | Weeks - semaines |
| **WWTP** | Wastewater treatment plant - station d'épuration |
| **XANES** | X-ray absorption near edge structure - Spectroscopie de structure près du front d'absorption de rayons X |
| **Zn** | Zinc |

# Chapitre I

# PROBLEMATIQUE DE L'ARGENT DANS L'ENVIRONNEMENT & OBJECTIFS DE CETTE THESE

L'industrialisation massive a permis à de nombreuses technologies de voir le jour ce dernier siècle, comme les nanotechnologies. Les nanotechnologies correspondent, d'après The Royal Society and The Royal Academy of Engineering (2004), à « la conception, la caractérisation, la production et l'application de structures, dispositifs et systèmes en contrôlant leur forme et leur taille à l'échelle nanométrique ». Ces dernières ont révolutionné tous les domaines de la physique à la biologie, en passant par la chimie, la médecine, l'ingénierie et l'électronique. La fabrication de masse de ces nanoparticules et produits nanofonctionalisés est alors devenue une nouvelle source de pollution. En effet, le cycle de vie de ces produits, de la fabrication à leur fin de vie, engendre la libération de nanoparticules dans l'environnement terrestre, aquatique mais aussi atmosphérique (Stampoulis et al., 2009; Yasur and Rani, 2013).

## 1) Contexte de la contamination par l'Ag et écotoxicologie de l'Ag et ses dérivés dans l'environnement terrestre

Les nanoparticules d'argent (AgNPs) sont un bon exemple de la contamination de l'environnement à cause de l'anthropisation. En effet, les AgNPs sont naturellement rares dans l'environnement. Hors, de nombreuses industries utilisent l'argent (Ag) et plus particulièrement les AgNPs pour exploiter ses nombreuses propriétés intéressantes, telles que, la plus connue, leur excellente action biocide (Chen and Schluesener, 2008; Fabrega et al., 2011; McGillicuddy et al., 2017; Wijnhoven et al., 2009) ou encore leurs propriétés de haute conductivité thermique

et électrique ou leur activité catalytique (Capek, 2004). Les produits nanofonctionnalisés libèrent parfois beaucoup d'Ag au cours de leur cycle de vie, avec notamment une voie de rejets majoritaires : les rejets dans les eaux usées (Donner et al., 2015). Le traitement des eaux usées au sein des stations d'épuration retient une grande partie de ces AgNPs dans ses déchets (Kaegi et al., 2011; Tiede et al., 2010). Un rapport de l'U.S. EPA (2009) indiquait que la concentration moyenne d'Ag dans ces boues d'épuration était de 20 mg $kg^{-1}$ mais les données de concentration variaient de 2 à 856 mg $kg^{-1}$ selon les boues analysées. De même, en Europe, la fourchette de concentrations de l'Ag dans les boues est estimée à 0,1-15 mg $kg^{-1}$ (Fijalkowski et al., 2017). Un rapport plus récent se focalisant sur les boues d'épuration en Chine montrait également une grande variabilité des teneurs en Ag d'une boue à l'autre avec des concentrations allant de 0,2 à 19 mg $kg^{-1}$ (Chen et al., 2020). Riches en matière organique, ces boues d'épuration sont souvent valorisées par l'épandage sur les sols agricoles. En Europe, entre 2010 et 2016, chaque année, environ 37 % des boues d'épuration produites ont été épandues (Eurostat, 2020), et ce chiffre peut doubler dans certains pays comme en Irlande ou Espagne (Eurostat, 2020; The Royal Society and The Royal Academy of Engineering, 2004). Les boues d'épuration étant riches en divers contaminants, des contrôles sont effectués régulièrement pour surveiller les teneurs en certains métaux ou composés organique (Directive européenne 86/278/CEE, 1986), cependant l'Ag n'est pas suivi, en Europe comme ailleurs (Rorat et al., 2019). Ainsi, les AgNPs d'origine anthropique se retrouvent en quantité considérable dans l'environnement terrestre. En 2010, on estimait qu'entre 80 et 190 tonnes d'Ag atterrissait dans les sols en Union Européenne (comprenant 25 pays à l'époque) via l'épandage de boues d'épuration (Blaser et al., 2008).

Du fait de ces rejets considérables d'argent dans l'environnement et de l'absence de contrôle de ce métal dans les boues d'épuration, l'Ag a fait l'objet de nombreuses études toxicologiques et écotoxicologiques, notamment ces 10 dernières années.

L'article de revue, intitulé "Ecotoxicology of silver nanoparticles and their derivatives introduced in soil with or without sewage sludge: A review of effects on microorganisms, plants and animals", propose une vision panoramique de la littérature actuelle au sujet des effets écotoxicologiques de l'argent et ses dérivés apportés par les boues d'épuration sur les sols.

Cette revue met en évidence quelques importantes conclusions. Les AgNPs peuvent réduire l'activité et l'abondance des microorganismes du sol ainsi que modifier leur communauté ; elles peuvent s'accumuler dans les végétaux et animaux ainsi qu'induire chez eux des effets biologiques. La taille, le revêtement, le substrat et évidemment leur concentration influencent leur toxicité. Les AgNPs agiraient en libérant des ions $Ag^+$ capables de pénétrer dans les organismes mais également, selon leur taille, en pénétrant tel quel au sein des organismes puis en y libérant des ions directement dans les cellules, c'est ce qu'on appelle l'effet « cheval de Troie » (Hsiao et al., 2015). Toutefois, les AgNPs intactes ne sont pas les molécules les plus intéressantes à étudier pour évaluer l'impact de l'Ag rejeté dans les sols via les boues d'épuration puisque ces nanoparticules sont transformées, au cours de leur passage dans les canalisations ainsi que lors des traitements des eaux usées puis des boues d'épuration. Ce sont ainsi des nanoparticules d'argent sulfuré ($Ag_2S$) principalement que l'on retrouve dans les boues. Les $Ag_2S$ ont été bien moins étudiés et leur éventuelle toxicité n'est pas encore bien éclaircie, toutefois les premières études montrent qu'elles sont davantage stables que les AgNPs et donc libèrent moins d'ions, ce qui engendre une moindre toxicité de manière globale pour les organismes. Néanmoins, les études manquent et n'ont pas toujours des résultats concordants, avec souvent des modes d'exposition peu réalistes en matière de durée d'exposition, concentration et substrat. Ainsi, la nécessité d'approfondir les connaissances sur la toxicité des nanoparticules d'Ag sulfurées apparaît comme une évidence, d'autant plus que la contamination des sols agricoles peut non seulement engendrer des accumulations dans les

chaînes trophiques mais aussi affecter la fertilité des sols, le rendement des cultures et ainsi l'économie agricole.

# Ecotoxicology of silver nanoparticles and their derivatives introduced in soil with or without sewage sludge: A review of effects on microorganisms, plants and animals.

Pauline Courtois[1], Agnieszka Rorat[1], Sébastien Lemiere[1], Rémy Guyoneaud[2], Eléonore Attard[2], Clément Levard[3] and Franck Vandenbulcke[1]

[1] *Univ. Lille - LGCgE - Laboratoire de Génie Civil et géo-Environnement, Cité scientifique, SN3, F-59655 Villeneuve d'Ascq, France*

[2] *Université de Pau et des Pays de l'Adour/E2S/CNRS, Institut des Sciences Analytiques et de Physico-chimie pour L'Environnement et les Matériaux (IPREM), UMR 5254, 64000, Pau, France*

[3] *Aix Marseille Univ, CNRS, IRD, INRA, Coll France, CEREGE, Aix-en-Provence, France*

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**Abstract**

Silver nanoparticles (AgNPs) are widely incorporated in many products, partly due to their antimicrobial properties. The subsequent discharge of this form of silver into wastewater leads to an accumulation of silver species (AgNPs and derivatives resulting from their chemical transformation), in sewage sludge. As a result of the land application of sewage sludge for agricultural or remediation purposes, soils are the primary receiver media of silver contamination. Research on the long-term impact of AgNPs on the environment is ongoing, and this paper is the first review that summarizes the existing state of scientific knowledge on the potential impact of silver species introduced into the soil *via* sewage sludge, from microorganisms to earthworms and plants. Silver species can easily enter cells through biological membranes and affect the physiology of organisms, resulting in toxic effects. In soils, exposure to AgNPs may change microbial biomass and diversity, decrease plant growth and inhibit soil invertebrate reproduction. Physiological, biochemical and molecular effects have been documented in various soil organisms and microorganisms. Negative effects on organisms of the dominant form of silver in sewage sludge, silver sulfide ($Ag_2S$), have been observed, although these effects are attenuated compared to the effects of metallic AgNPs. However, silver toxicity is complex to evaluate and much remains unknown about the ecotoxicology of silver species in soils, especially with respect to the possibility of transfer along the trophic chain *via* accumulation in plant and animal tissues. Critical points related to the hazards associated with the presence of silver species in the environment are described, and important issues concerning the ecotoxicity of sewage sludge applied to soil are discussed to highlight gaps in existing scientific knowledge and essential research directions for improving risk assessment.

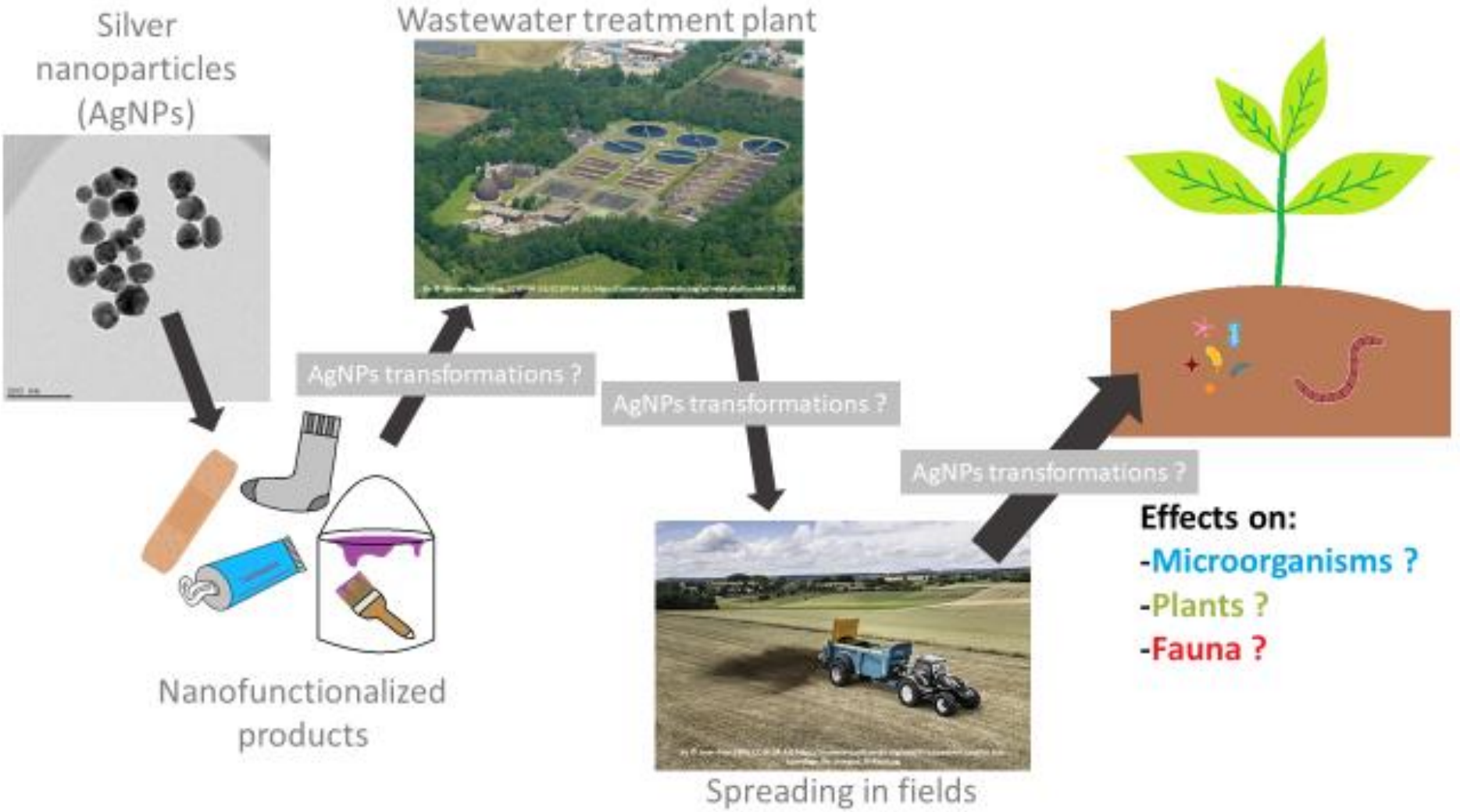
**Keywords**

Silver nanoparticles, ecotoxicology, sewage sludge, terrestrial ecosystem, silver speciation

**Capsule**

The terrestrial ecotoxicity of silver nanoparticles is the result of complex interplay between the properties of the silver species and the receiver medium.

**Graphical abstract**



## 1. Introduction

Nanotechnologies allow the "design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer scale" (The Royal Society and The Royal Academy of Engineering, 2004). A nanomaterial is a "material with any external dimensions in the nanoscale or having internal structure or surface structure in the nanoscale", i.e., between 1 nm and 100 nm (European commission, 2012; ISO/TS 80004-1:2015). Technological progress in nanotechnologies since the second half of the 20th century might have engendered new contaminations of the environment due to the widespread use of manufactured nanoparticles (NPs), which are highly abundant and diversified. NPs are found in all compartments of the environment: atmosphere, water and soil (Gottschalk et al., 2013; Lead et al., 2018; Yasur and Rani, 2013).

Silver NPs (AgNPs) illustrate the potential problem of environmental contamination by anthropogenic NPs. Silver (Ag) is a naturally occurring rare metal that is 63$^{rd}$ in order of abundance of chemical elements in Earth's crust. In the environment, Ag can form numerous complexes of varying solubility by associating with anions such as chloride or sulfur (Levard et al., 2013, 2011). Silver has been used for centuries due to its antimicrobial properties, with anecdotal evidence of the use of colloidal silver in ancient Egypt and Rome (Reidy et al., 2013). Silver continues to be used in several forms to preserve fluids (such as water) or treat various aliments (Castellano et al., 2007; Nowack et al., 2011).

The large-scale manufacture of consumer products that contain AgNPs results in significant release of Ag in the environment. Although the amount of produced AgNPs is smaller compared to the production of other NPs (like $TiO_2$-NPs and $SiO_2$-NPs, INERIS, 2016) , it remains substantial and its toxicity potential is high. Still, the world AgNPs production is between 135 and 420 tons/year (INERIS, 2016).

Although AgNPs are primarily known for their powerful antimicrobial (bactericidal) effects, only 10% of manufactured AgNPs are intended for antimicrobial use (European commission, 2012). Other applications of AgNPs include uses in electronics, optics, biosensing and catalysis (European commission, 2014). Given the ubiquity of AgNPs, their release into the environment as part of the life cycle of AgNP-containing products may have significant impacts on ecosystems and/or human health (ANSES, 2015; Rui et al., 2017; Wijnhoven et al., 2009). Consequently, numerous studies have sought to assess hazards and risks to organisms and ecosystems related to the use of engineered AgNPs and their derivatives.

Soil is the main repository of Ag through land application of post-treated organic wastes, e.g., sewage sludge (SS) (Massarsky et al., 2014). Notably, AgNPs undergo important environmental transformations, including sulfidation, in wastewater treatment plants (WWTPs) (Levard et al., 2012) that can strongly alter their behavior in soils compared to directly applied AgNPs used in ecotoxicity assessment works. However, most studies of silver ecotoxicology have been conducted in aquatic environments (Fabrega et al., 2011; McGillicuddy et al., 2017; Zhang et al., 2016). Research studies focusing on terrestrial environments are increasing, but a comprehensive overview of the fate, behavior and effects of Ag in this complex matrix is lacking. Several reviews exist on effects of AgNPs and their derivatives but these are often very general (like Yu et al., 2013). Here, we propose a detailed study about toxicological and ecotoxicological effects of the AgNPs, but also species derived from Ag like silver sulfides, in terrestrial environments, major receptacle of these contaminants. Thus, the aim of this review is to discuss existing knowledge on the fate and ecotoxicology of Ag in different forms (ionic, NPs or $Ag_2S$) in soils and its impacts on 3 groups of organisms: microorganisms, plants and animals.

## 2. Current silver release into the environment

### 2.1. Development and use of AgNPs

Advances in the nanotechnology industry in the 1990s ultimately led to exponential growth of production of nanomaterials in subsequent years (Hullmann, 2006). Many types of AgNPs differing in size and shape are manufactured. The majority have a core-shell structure comprising a metallic silver core of varying size and shape and a coating that usually helps to control a size of the AgNPs during synthesis and provides a surface charge to stabilize the AgNPs in solution (Levard et al., 2012). The many interesting properties of AgNPs allows for a wide range of applications. Their small size confers a high surface area/volume ratio that allows high contact with microorganisms. AgNPs are therefore powerful, wide-spectrum antibacterial agents (Bone et al., 2012; Maillard and Hartemann, 2013; Morones et al., 2005; Reidy et al., 2013). At the beginning of the 20th century, wounds and infections were sometimes treated with colloidal silver (including AgNPs), but the development/discovery of modern antibiotics in the 1940s greatly reduced this practice (Reidy et al., 2013). With the increasing resistance of bacteria to antibiotics, Ag has again become popular in the medical field as a disinfectant (Rai et al., 2009). AgNPs also have many distinctive physicochemical properties, such as high electrical and thermal conductivity, catalytic activity, improved surface Raman scattering and non-linear optical behavior (Capek, 2004). Thus, a diverse array of nanofunctionalized consumer products contains AgNPs: textiles and clothing, medical devices, household appliances such as washing machines and refrigerators, food packaging, cosmetics, detergents, paints, plastics, electronic circuitry, water filters and biosensors (Gottschalk et al., 2013; McGillicuddy et al., 2017). AgNPs are not the most-produced nanoparticle in terms of quantity (Vance et al., 2015) but, for all of the reasons cited above, are the most widespread nanomaterial; in 2013, 435 of 1835 referenced nanomaterial-containing products included AgNPs (Vance et al., 2015).

### 2.2. Route of release – the importance of wastewater treatment plants

Before the arrival of modern nanotechnology, environmental Ag was primarily the result of the natural pedo-geochemical background, natural leaching, and two anthropogenic causes: mining activity and the photographic industry (Fabrega et al., 2011). The latter source of anthropogenic contamination has been largely eliminated by the rise of digital photography. However, the use of AgNPs in common consumer products represents an emerging source of contamination. The synthesis of nanomaterials and the manufacture, distribution, regular use, and end-of-life elimination of nanofunctionalized products are possible routes of environmental contamination (McGillicuddy et al., 2017). Kaegi and colleagues estimated that more than 30% of the Ag in paint on a panel of a house facade is released directly into the environment in the first year (Kaegi et al., 2010). Indirect domestic releases should also be considered, such as washing of nanofunctionalized textiles. Depending on the quality of manufacture and the type of washing, textiles can release between 48 and 94% of Ag during the first 20 washes (Limpiteeprakan et al., 2016).

It is difficult to quantify the release of manufactured AgNPs in the environment, but the wastewater network has been shown to be a key route of release of AgNPs and their derivatives in the environment (Donner et al., 2015). A modeling study showed that 37 - 46% of produced AgNPs ends up in the sewer system (Adam et al., 2018). At realistic Ag concentrations, around 90% of Ag is held in biosolids (Kaegi et al., 2011; Tiede et al., 2010). The quantities found in biosolids can vary considerably from one WWTP to another, depending on the nature of the surrounding activities (Gottschalk et al., 2013). In 2013, the estimated average concentration of Ag in biosolids was 1 mg $kg^{-1}$ of dry matter (Gottschalk et al., 2013). However, in 2009, a report by the EPA (U.S. EPA, 2009) indicated Ag concentrations ranging from approximately 2 to 856 mg $kg^{-1}$, with a mean of 20 mg $kg^{-1}$. The importance of wastewater release highlights the need to monitor the fate and behavior of Ag in WWTPs and SS. Moreover, to exploit its high

content of organic matter and compounds such as phosphorus and nitrogen, SS is spread ("valorized") on agricultural fields in many European countries. In Europe, 45% of the total quantity of SS is recycled this way (European commission, 2017), and this number can approach 80-90% in some countries, such as Portugal and Ireland (data from 2012 and 2015, Eurostat). Given that 90% of Ag is accumulated in biosolids, the amount of Ag that passes through WWTPs and is returned to the environment *via* sludge is potentially considerable. In an analysis of scenarios of the release of engineered silver into the environment, Blaser et al. (2008) estimated that 140 tons of Ag enters agricultural soil annually from spreading of SS. Adam et al. (2018) showed that among all produced AgNPs, 17 - 22% landed in soils, 7 - 16% in water, 3 - 4% in air and 48 - 58% were recycled / incinerated / landfilled.

### 2.3. Transformation of silver in WWTPs and effects on its properties

Studies of the behavior of AgNPs in WWTPs have shown that silver undergoes transformation, and the predominant Ag species following wastewater treatment is silver sulfide ($Ag_2S$) (Kaegi et al., 2013, 2011; Kent et al., 2014; Levard et al., 2012; Lombi et al., 2013; Ma et al., 2014; Pradas del Real et al., 2016). Anaerobic bacteria in sewers and WWTPs produce sulfides (Kaegi et al., 2013) that can react with Ag due to the strong affinity between these two molecules (Levard et al., 2012). The surface functionalities (coating) of NPs do not inhibit the transformation of Ag within a WWTP, and the Ag is ultimately mostly sulfidized (Lombi et al., 2013). Levard et al. (2011) showed that sulfidation of AgNPs affects the state of aggregation of particles (despite the polyvinylpyrrolidone (PVP) coating usually used to limit this phenomenon) and the surface charge of the particles. Most importantly, $Ag_2S$ is one of the least soluble Ag species, and thus its formation potentially lowers the toxicity of Ag in the environment by decreasing the release of Ag+ ions (Levard et al., 2013, 2011; Nowack et al., 2011). In addition, $Ag_2S$ is very stable over time; Lombi et al. (2013) showed that $Ag_2S$ did not

undergo transformation under biosolid composting/storage conditions (duration of 6 months), unlike other metals such as zinc and copper. Similarly, an analysis of numerous biosolid samples with different degrees of aging demonstrated significant concentrations of $Ag_2S$, suggesting stability of these molecules over several years/decades (Donner et al., 2015). However, studies demonstrated that even if Ag is completely sulfided, a portion (60%) of $Ag_2S$ is poorly crystalline (amorphous $Ag_2S$) and has a higher degree of solubility (Kampe et al., 2018; Kraas et al., 2017; Levard et al., 2011). In addition, $Ag_2S$ stability can be affected by oxidizing agent naturally present in the environment like $MnO_2$, which favors $Ag_2S$ dissolution (Shi et al., 2018). So, sulfidation of AgNPs is not always complete, and dissolution of Ag, although attenuated, can still occur (Baalousha et al., 2015; Kent et al., 2014).

Thus, because of this new income of Ag in terrestrial environments, Ag deserves to be studied like an emerging contaminant, at least "re-emerging". There are two important reasons for the need to continue the deepen studies on Ag. The first is the possible transfer along the trophic chain (Fabrega et al., 2011; Wu et al., 2018); i.e. silver-contaminated SS is being spread on agricultural soils where crop species are cultivated which could directly affect animal and human health. The second reason is the potential for speciation of Ag. Silver undergoes physicochemical transformations such as oxidation, dissolution, sulfidation and aggregation (Domingos et al., 2009; McGillicuddy et al., 2017), partly controlled by soil properties. These transformations can affect its impact and transfer in the environment.

Classic ecotoxicological studies have assessed the effects of ionic silver, AgNPs and, rarely, sulfidized silver in simplified or realistic experimental systems. These studies have analyzed organisms conventionally considered in soil ecotoxicology: microorganisms, fungi, algae, terrestrial plants and soil invertebrates. These organisms have critical functions in the soil ecosystem and, most importantly, are all likely to be directly influenced by contaminants in the soil due to their close contact with the soil during feeding, reproduction, and growth.

Many initial studies of the toxicity of AgNPs on organisms employed simplified matrices or simply added AgNPs directly to soil. Such studies are appropriate for revealing the impact of direct environmental exposure to AgNPs *via* atmospheric deposition, landfills and accidental spills. However, more than 90% of silver entering WWTPs is partitioned into SS (Kaegi et al., 2011; Kraas et al., 2017; Tiede et al., 2010). Silver concentrations in wastewater do not appear to affect the operation of stations significantly (Rasool and Lee, 2016), and AgNPs, which are mainly transformed to $Ag_2S$, can be transported into the environment, mainly *via* SS spreading on agricultural land (Kaegi et al., 2013, 2011). Therefore, studies mimicking SS spreading on soils have been performed to reflect a more realistic route of entry of silver in soils and to observe the effects of $Ag_2S$ in the environment. In the following sections, we discuss the effects of Ag on microorganisms, plants and, finally, invertebrates. Each section will begin by introducing a few studies of the effects of directly supplied silver (without WWTP treatment or simulation) for comparison with recent studies integrating a realistic route of entry *via* WWTPs.

## 3. Effects of silver on soil microorganisms

### 3.1 *In vitro* effects of AgNPs on microorganisms

Although AgNPs may undergo transformation before arriving in the soil compartment, the bactericidal properties of ionic silver suggest a potential for negative effects on soil microbial communities (Table 1). Laboratory experiments with pure cultures (Fig. 1) have confirmed growth-inhibiting and bactericidal effects of silver (AgNPs and $Ag^+$) on ecologically important bacteria such as *Nitrosomonas europaea*, *Nitrosospira multiformis*, and *Bacillus subtilis* (Beddow et al., 2014; Michels et al., 2015). Enriched cultures of ammonia oxidizing bacteria exposed to silver exhibit greatly reduced nitrifying activity, and this decrease is particularly pronounced after exposure to AgNPs compared to $Ag^+$ ions (Choi et al., 2008; Michels et al., 2017, 2015). These deleterious effects have been attributed to the disruption of

membranes by interactions with free silver ions or protein inactivation due to interactions with thiol functional groups (Morones et al., 2005). This also could be partly explained by the increased production of reactive oxygen species that accumulate and damage cellular constituents (Choi and Hu, 2008). As an example, in the nitrifying bacterium *Nitrosomonas europaea,* Barker et al. (2018) observed enzymatic inhibition at low concentrations of silver and cell death at high concentrations. Although providing interesting information about mechanisms of AgNPs toxicity, these studies were performed *in vitro* or, in other words, simplified systems comprising synthetic media and bacteria in a particular physiological state, with no or extremely reduced interactions with other organisms and environmental parameters. Thus, extreme caution is required when extrapolating the results of these studies to soil microbial communities. Studies with realistic experimental designs and *in situ* and mesocosm experiments that consider reasonable concentrations and representative exposure scenarios are needed.

### 3.2. In situ effects of direct exposure of AgNPs on microorganisms

The responses of microbial communities to a disturbance such as AgNPs supply can be characterized based on the effects on 3 types of parameters: abundance, diversity/structure and activity rate. In this section, we synthesize the results of works categorized according to these parameters.

#### 3.2.1. Abundances and structures of microbial communities

Most studies have shown a decrease in the abundance of the total microbial community linked to the dose of AgNPs applied directly on soil (Hänsch and Emmerling, 2010; He et al., 2016; Samarajeewa et al., 2017) (Fig. 1). Similarly, nitrifier community abundances are decreased (McGee et al., 2017; J. Wang et al., 2017) more intensely after AgNPs supply compared to ionic silver exposure (He et al., 2016). Strikingly, Grün et al. (2018) observed

increasing toxicity over one year, i.e., no effects on the abundance of nitrifiers in the first few months but negative effects after one year. The authors hypothesized that this pattern is attributable to long-term release of $Ag^+$ from AgNPs. Although few long-term studies are available, these results highlight the need to integrate changes in AgNPs over time and their potential influences on toxicity.

Similarly, to the effects on abundance, direct exposure to AgNPs alters the structure of bacterial communities (Kumar et al., 2014; Liu et al., 2017; McGee et al., 2017; Sillen et al., 2015) notably in a dose-dependent way (Samarajeewa et al., 2017; J. Wang et al., 2017) and with increasing toxicity with time (Grün and Emmerling, 2018). The patterns of response of the structure of fungal communities are less straightforward. McGee et al. (2017) and Sillen et al. (2015) showed that the structures of both fungal and bacterial communities are affected by AgNPs. However, Kumar et al. (2014) observed weaker effects on the structures of fungal communities compared to bacterial communities. The authors of these studies did not elucidate the underlying mechanisms but suggested that these differences were due to greater resistance of fungi to AgNPs and/or outcompetition of bacteria for resources to survive. The changes in structure are due to changes in species/operational taxonomic unit (OTU) composition linked to a decrease in diversity indexes (richness, Shannon and Simpson's indexes) (Grün and Emmerling, 2018; Liu et al., 2017; Samarajeewa et al., 2017). Although the abundances of some phyla decrease significantly after exposure, no clear trend has been validated across different studies, thus preventing the definition of a proxy of AgNPs contamination. With respect to particle size, Kumar et al. (2014) observed a higher impact of nanoparticles compared to microparticles on the structures of bacterial communities, whereas Doolette et al. (2016) observed no effect of particle size.

### 3.2.2. Microbial activities

Activity rates are relevant parameters linked to soil functionality and are by far the most-studied parameters in the literature (Fig. 1). Studies have primarily focused on processes performed by most of soil microorganisms, such as carbon respiration or dehydrogenase or urease activities. Most studies to date have shown significant decreases in these processes after spiking with AgNPs (Peyrot et al., 2014; Rahmatpour et al., 2017; Schlich et al., 2016; Schlich and Hund-Rinke, 2015; Shin et al., 2012). Only Hänsch and Emmerling (2010) did not observe any effect on respiration until 0.32 mg $kg^{-1}$ of AgNPs and they explain that this corresponds to a cryptic response. Thus, the AgNPs introduced directly into the soil have a clearly negative impact on microbial activities at concentration of 1.6 mg $kg^{-1}$ and higher. However, depending on the soil type and the studied activity, it is possible to observe a negative effect even at 0.01 mg $kg^{-1}$. Positive dose-response relationship is not always observed and soil properties condition such effects. Despite decreases in activity rates observed in different soil types, Schlich and Hund-Rinke (2015) and Rahmatpour et al. (2017) suggested a link between clay content and the effects of AgNPs, with lower clay content leading to a higher impact on soil respiration. The dependence of the response on soil type has received little attention and warrants further study.

A number of studies (Peyrot et al., 2014; Rahmatpour et al., 2017; Shin et al., 2012) have reported that AgNPs are more toxic to microbial activities than ionic silver in exposed bacterial communities. Variations in the size, shape, and coatings of AgNPs represent another level of complexity in studying their effects that hinders direct comparisons between studies. Indeed, Zhai et al. (2016) showed that nanorods and nanoplates exhibit greater toxic effects on the ability to degrade carbon substrates than nanospheres. However, several other studies have shown decreased activity rates regardless of the type of AgNPs and coating (Peyrot et al., 2014; Rahmatpour et al., 2017; Schlich et al., 2016; Schlich and Hund-Rinke, 2015; Shin et al., 2012).

Almost half of the studies showing effects of AgNPs on processes performed by many microbial taxa have also measured a process performed by microbial taxa characterized by a lower level of diversity and lower abundances, such as the nitrifying community. This complementary approach can provide a better assessment of the overall impact on microbial communities. This type of process that is performed by a few microbial taxa, less-diverse and less-abundant, could be affected in a very different way than a process performed by a huge diversity of microorganisms with high abundance. In addition, a process such as nitrification is a crucial step in the nitrogen cycle, its response to AgNPs is thus particularly important in agroecosystems. Overall, AgNPs negatively affect nitrification (He et al., 2016), and some studies have revealed different responses of nitrification and respiration. Samarajeewa et al. (2017) showed a dose-dependent response for respiration but a hormetic response for nitrification. In the study of Schlich et al. (2016), a single application of AgNPs affected nitrification more intensely than repeated application, but opposite results were obtained for respiration. These non-homogeneous responses illustrate that the effects of AgNPs can vary depending on the microbial communities; therefore, it is not easy to generalize results obtained for one community to the functionality of the whole ecosystem.

Studies on the effects of direct exposure to AgNPs have consistently highlighted decreases in microbial activities linked to changes in microbial community structure and decreases in abundance regardless of soil type and with a weak importance of AgNPs size and shape. The contradictory results observed between some studies are mostly attributable to (i) effects on one specific microbial community that do not apply to all others; (ii) the complexity of microbial community structure as a variable for interpretation; (iii) the diversity of experimental designs, including the dose and nature of AgNPs, soil type, and experiment duration. Above all, since direct exposure is not a realistic route of entry of AgNPs in soil ecosystems, more studies mimicking SS spreading on soils are needed.

### 3.3. In situ effects of AgNPs supplied *via* sewage sludge on microorganisms

Only six studies have been designed to assess the effects of AgNPs supplied *via* SS (Asadishad et al., 2018; Colman et al., 2013; Durenkamp et al., 2016; Kraas et al., 2017; Samarajeewa et al., 2019; Schlich et al., 2017), and two others have studied the effects of a supply of $Ag_2S$-NPs to mimic the sulfidation process occurring during SS fermentation (Doolette et al., 2016; Judy et al., 2015).

#### 3.3.1. Abundances and structures of microbial communities

No effects of $Ag_2S$-NPs on microbial biomass (Judy et al., 2015) and nitrifier abundance were observed at low silver concentrations (Doolette et al., 2016). At higher silver concentrations (10 to 100 mg $kg^{-1}$), the abundance of nitrifiers decreased more intensely after introduction of AgNPs compared to $Ag_2S$-NPs, confirming that sulfidation of nanoparticles decreases their toxicity (Doolette et al., 2016). Consistent with these changes in abundance, the structures of the microbial communities did not exhibit major changes (Doolette et al., 2016; Durenkamp et al., 2016). However, contradicting these results, another study observed a decrease in microbial biomass and a change in the structure of the microbial community as soon as one day after exposure with 0.14 mg $kg^{-1}$ of sulfided Ag (Colman et al., 2013). In that study, the authors spiked AgNPs in the SS immediately before mixing with soil. Although silver sulfidation usually occurs rapidly in sludge, the lack of fermentation of the SS may have hindered the sulfurization of silver, and only 25% of silver was present in the less toxic $Ag_2S$ form. Moreover, the structure of the microbial community recovered within 50 days, confirming that sludge aging is critical for sulfidation and reduced toxicity.

#### 3.3.2. Microbial activities

After application of SS spiked with AgNPs, Kraas et al. (2017), Schlich et al. (2017), Schlich et al. (2018) and Samarajeewa et al. (2019) observed a continuous decrease in

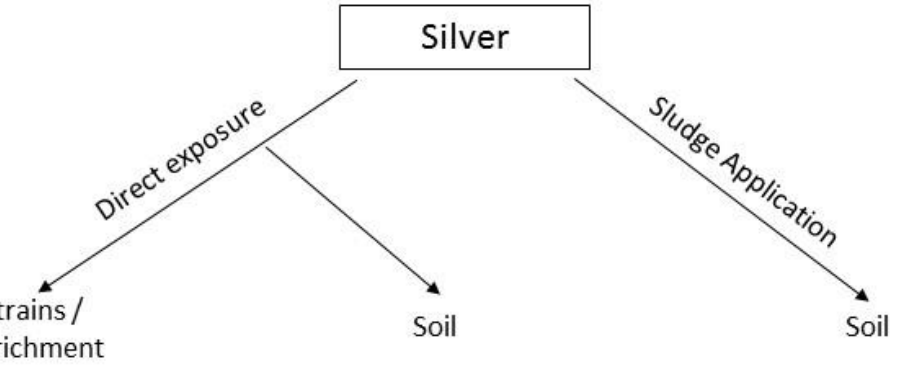
nitrification activity compared to the control up to 25 months. The activity of four extracellular enzymes from the nutrient cycle (cellobiohydrolase, xylosidase, acetylglucosaminidase, acid phosphatase; out of 5 tested) was still impacted after 30 days of incubation in the highest concentration (100 mg $kg^{-1}$) (Asadishad et al., 2018). In addition, these studies provide two striking conclusions: an increase in toxicity over time and a strong effect of even $Ag_2S$ on some microbial activities. The temporal pattern of response can be explained by soil retention of silver and subsequent slow release over time. Although Levard et al. (2013) showed that $Ag_2S$ is less toxic than AgNPs, it negatively affects higher organisms (zebrafish, killifish, nematodes, and duckweed). Others authors have observed no effects of $Ag_2S$ NPs on respiration activity and $CO_2$ and $N_2O$ fluxes (Doolette et al., 2016). Therefore, the different responses of the various communities (nitrifiers or C oxidizers) are likely due to differences in physiology and, in turn, levels of functional redundancy.

### 3.4. Overview of the effects on microorganisms

In summary, the vast majority of studies have shown that direct exposure to AgNPs leads to a decrease in microbial activity rates that is frequently accompanied by a decrease in microbial abundance or a change in the structures of microbial communities. By comparison, AgNPs exposure through SS application engendered more moderate effects on microbial communities, but such works remain scarce and over time the negative effects of sulfided AgNPs are significant. The toxicity does not depend on the exposure concentration. Some taxa or activities may respond more to an average dose than to lower or higher doses. Others may respond in a dose-dependent manner in one context but not in another. Microbial communities are so variable from one soil to another that current results are difficult to generalize. Carefully designed experiments adopting realistic conditions for SS application, including longer time scales, repeated applications, and different soil types, are needed. Studies to date have mainly

targeted the structure and activity of the total microbial community and largely omitted characterizations of abundance. Targeting microbial communities with different requirements and focusing on different characteristics of these communities would provide a more accurate assessment of how microorganisms respond to AgNPs in soil and their impact on the functioning of the whole soil ecosystem.

AgNP: silver nanoparticle
AgMP: silver microparticle



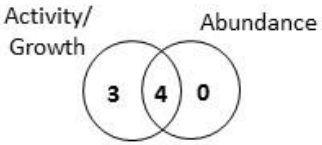
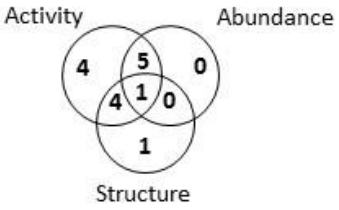
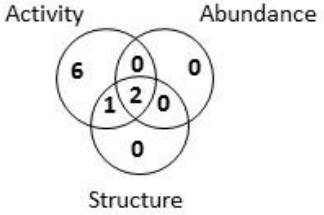

| | | | | | | |
|---|---|---|---|---|---|---|
| **Number of studies** | | 7 | 15 | | 9 | |
| **Concentration range** | | 0.05 - 50mg.$L^{-1}$ | 0.001 - 5590 mg.$kg^{-1}$ | | 0.56 - 706 mg.$kg^{-1}$ | |
| **Duration range** | | 30 min - 7 days | 2h - 365 days | | 8 days - 180 days | |
| **Repartition of studies depending on the microbial parameters measured** | |   |   | |   | |
| **Summary of main results** | | Response Type | Response Type | Intensity of effects | Response Type | Intensity of effects |
| | Activity | ↘ | ↘ or = or hormetic | AgNP > Ag+ | ↘ or = | |
| | Abundance | ↘ | ↘ | AgNP > Ag+ | ↘ or = | |
| | Structure | | Change | AgNP ≥ $Ag_2S$/AgMP/Ag+ | Change | $Ag_2S$ < AgNP/Ag+ |

Figure 1: Schematic overview of available studies on AgNP effects on microorganisms

*Table 1: Overview of key studies focusing on the effects of AgNPs on microorganisms*

| Targeted organisms | Matrix | Form and size of AgNPs | Concentration / Dose | Exposure Time | Main Effects | Reference |
|---|---|---|---|---|---|---|
| Gram negative bacteria | Enrichment culture | Data not shown | 0 to 100 mg/L | 30 m | AgNPs disrupt membrane functions (like permeability and respiration) of bacteria by attaching to the surface. AgNPs can penetrate inside bacteria and cause damage by interacting with DNA. The toxicity depends on the size of the AgNPs (only small AgNPs interact directly with bacteria). | Morones et al. (2005) |
| Nitrifying bacteria and *Escherichia coli* | Enrichment culture | PVA-coated (16 nm)/AgCl | 0.1 to 1 mg/L | 24 h | AgCl colloids inhibit respiration and nitrification (lack of change in dissolved oxygen) as well as AgNPs; the effect varies depending on the size and bioavailability of the NPs. Ag does not affect the integrity of membranes. | Choi et al. (2008) |
| Ammonia-oxidizing bacteria (AOB) and *Escherichia coli*, *Bacillus subtilis* | Culture | Non-coated AgNPs (118 ±11 nm), mPEG-coated AgNPs (27 nm), $Ag_2SO_4$ | 0.5 to 50 mg/L | 14 h | All silver treatments affect AOB, reducing nitrification potential rates. The growth of *E. coli* and *B. subtilis* is strongly affected by $Ag_2SO_4$. Globally, $Ag_2SO_4$ is more toxic than-coated AgNPs, which are more toxic than non-coated AgNPs. The size of the AgNPs probably plays a role in toxicity differences. | Beddow et al. (2014) |
| *Nitrosomonas europaea* | Culture | Amorphous carbon-coated AgNPs (35 nm)/$AgNO_3$ | 0.075 to 0.75 mg/L | 1,3,5,7 d | AgNPs reduce the rates of $N_2O$ production, ammonia oxidation and NO2 production by regulating the expression of some genes (nitric oxide reductase, ammonia monooxygenase and nitrite reductase). | Michels et al. (2015) |
| *Nitrosomonas europaea* | Culture | AgNPs (25.5 nm)/ $Ag^+$ | 0.1 mL/h of solution 0.05 to 2 ppm $Ag^+$ 1.5 to 20 ppm AgNPs | 3-48 h | AgNPs cause enzymatic inhibition at low concentrations and cell death at high concentrations. | Barker et al. (2018) |
| Ammonia-oxidizing bacteria (AOB) | Culture | AgNPs (45 nm) | 0 to 30 mg/L | 14 h | AgNPs reduce the AOB-specific nitrite production rate. Nanoparticles attach to the bacterial surface, modify membrane permeability and disrupt AOB activity. | Michels et al. (2017) |
| Ammonia and nitrite oxidizing bacteria | Suspension | PVA-coated (15 ± 9 nm) | 9 nM | 18 h | Ag inhibits nitrification. Sulfidation reduces the toxicity of AgNPs on nitrification by 80%. | Choi et al. (2008) |
| Heterotrophic bacterial community | Natural sandy loam soil | Data not shown | 0.0032 to 0.32 mg /kg | 120 d | AgNPs cause a decrease in carbon microbial biomass and increase the metabolic quotient. | Hänsch and Emmerling (2010) |

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| Soil microbial community | Loamy soil | BAM-N001 (20 nm)/ $AgNO_3$ | 0.01 mg/kg | 1 y | AgNPs cause a decrease in biomass and the activity of soil microorganisms. The toxicity increases over time (possibly linked to $Ag^+$ release). | Grün et al. (2018) |
| Soil bacterial phyla | Loamy soil | BAM-N001 (20 nm)/ $AgNO_3$ | 0.01 mg/kg | 1 y | AgNPs cause a reduction of several phyla of bacteria upon long-term exposure, affecting important functions of soil like nitrification or organic carbon transformation. | Grün and Emmerling (2018) |
| Soil microbial community | Natural soil | Citrate-coated (10 nm)/$AgNO_3$ | 1, 10, 100 and 1000 mg/kg | 7 d | AgNPs decrease the activity of soil exoenzymes related to nutrient cycles (urease, acid phosphatase, arylsulfatase, β-glucosidase) and overall microbial activity (dehydrogenases, fluorescein diacetate hydrolases). | Shin et al. (2012) |
| Soil microbial community | Natural sandy soil | Polyacrylate-coated (2-10 nm)/$AgNO_3$ | 0.001-0.1 mg/kg, 1-30 mg/kg, 750 mg/kg | 5 w | AgNPs have a negative effect on soil enzyme activities (soil enzymes: hydrolases), especially at low concentrations. AgNPs release few ions. | Peyrot et al. (2014) |
| Bacterial and fungal assemblages | Natural soil | AgNPs (20nm)/AgMPs (3000 nm) | 0.066% and 6.6% | 71 d | Small particles are more toxic (decrease in levels of respiration, decrease in signature bacterial fatty acids, changes in richness and evenness in bacterial and fungal DNA sequence assemblages). | Kumar et al. (2014) |
| Heterotrophic bacterial and nitrifying communities | Loamy sand | NM-300K (15 nm) | 1.67 and 5 mg/kg supplied in one or three applications | 28,56,84 d | Single application has a stronger effect on potential nitrification than the same dose applied in 3 doses, whereas an opposite pattern is observed for potential respiration. | Schlich et al. (2016) |
| Soil microbial community | Natural soil | $Ag_2S$-NPs/AgNPs/$AgNO_3$ | 0.1 to 93 ($AgNO_3$), to 404 (AgNP), to 5590 mg/kg ($Ag_2S$) | 28 d | The 3 forms of Ag decrease nitrate production in a dose-dependent way. The changes in the composition of the soil microbial community vary according to the Ag form. Some organism families are more sensitive to Ag (like Bacillaceae). | Doolette et al. (2016) |
| Soil microbial community | Suspension (with soil extract) | AgNPs (between 15 - 80 nm) /$AgNO_3$ | 0.07 to 1.529mg/L (AgNP)<br>0.003 to 0.05 mg/L ($AgNO_3$) | 0,24,48,72,96 h | The diversity of microorganisms decreases with silver. The functional composition of microbial communities is dependent on the AgNP forms. $AgNO_3$ is more toxic than AgNP. | Zhai et al. (2016) |
| Soil microbial community | Natural soil | PVP-coated | 60 to 2000 mg/kg | 63 d | The growth and activity of microorganisms are affected by low concentrations of silver.<br>*Rhodanobacter sp.* seems to be tolerant to silver. | Samarajeewa et al. (2017) |
| Soil microbial community | Natural soil | AgNPs (20 ± 3 nm) | 0.1, 1, 10 mg/kg | 30 d | AgNP amendents cause decreases in soil microbial metabolic activity, nitrification potential and the abundances of bacteria and ammonia-oxidizing bacteria. | He et al. (2016) |

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| Soil microbial community | Natural soil | Citrate-coated (9-10 nm) | 1 mg/kg | 2,7,21,35,49 d | A low dose of AgNPs affect the structure of the soil bacterial community provisionally (recovery after 49 days). The soil microorganism communities change during different plant growth stages. | Liu et al. (2017) |
| Heterotrophic bacterial and nitrifying communities | Natural soil (pasture soil) | AgNPs (20 ± 10 nm) | 50 mg/kg | 3,6,10,20,30 d | AgNPs reduce dehydrogenase and urease within 3 days and throughout the experiment duration, decrease the abundance of nitrifiers, and change the soil bacterial and fungal communities. | McGee et al. (2017) |
| Soil microbial community | Natural soils | PVP-coated (40-45 nm) / $AgNO_3$ | 0.01 to 50 mg/kg | 7-60 d | Low doses of silver have no effect on soil respiration or urease and phosphatase activities, as opposed to high doses. | Rahmatpour et al. (2017) |
| Soil microbial community | Natural soil | AgNPs (50 nm) | 10,50, 100 mg/kg | 7 d | AgNPs inhibit the growth of *E. coli.* Sulfidation reduces the negative effects of AgNPs, which depend on the initial size (polydispersity) of the AgNPs and their aggregation state. | Wang et al. (2017) |
| Heterotrophic bacterial community | Coarse loamy soil in mesocosms with plants *in situ* | PVP-coated (21 ± 17 nm)/$AgNO_3$ supply via sludge | 0.14 mg/kg (AgNP) 0.56 mg/kg ($AgNO_3$) | 8,50 d | AgNPs have negative effects on potential nitrification, but significant effects are also reported for $AgNO_3$. | Colman et al. (2013) |
| Microbial phospholipid fatty acids (PLFA) | Loamy sand + sludge | $Ag_2S$ / PVP AgNPs / $AgNO_3$ | 1,10,100 mg/kg | 8 w | Sulfided AgNPs and $Ag^+$ do not affect microbial biomass and respiration but decrease the Gram+/Gram- ratio, suggesting a change in the composition of the microbial community. | Judy et al. (2015) |
| Soil microbial community | 5 RefeSol + sludge | NM-300K (15 nm)/ $AgNO_3$ | 0.19 to 15 mg/kg | 1-28 d | Sulfided AgNPs reduce the potential respiration in some soils at the highest concentration and potential nitrification in all soils even at weak concentrations. | Schlich and Hund-Rinke (2015) |
| Soil microbial community | Natural soil + sludge | PVP-coated (52 nm) | 3 mg/kg | 180 d | Metals inhibited nitrification at the beginning of the experiment. No major changes were observed in the structure of the microbial community (just reduction of the fungal component). | Durenkamp et al. (2016) |
| Soil microbial community | Natural soil + sludge | NM-300K (15 nm) | < 9 mg/kg | 140 d | Sulfided AgNPs remain immobile in soil. Potential nitrification and respiration are reduced at the highest AgNP concentration even at the end of the long-term experiment. | Kraas et al. (2017) |
| Heterotrophic bacterial and nitrifying communities | RefeSol01A + sludge | NM-300K (15 nm)/$AgNO_3$ | 1.8 and 7.0 mg/kg | 180 d | Soil microflora are constantly inhibited for 25 months at a high concentration of sulfided AgNP (low activity of ammonium oxidizing bacteria and low respiration activity). | Schlich et al. (2017) |

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| Soil microbial community | Natural soil + sludge | Citrate-coated (50 nm)/$Ag^+$ | 1 to 100 mg/kg | 30 d | Sulfided AgNPs inhibits enzymatic activities at different concentrations depending on the activity. | Asadishad et al. (2018) |
| Soil microbial community | RefeSol01A + sludge | NM-300K (15 nm) /$AgNO_3$/ $Ag_2S$ NM-300K/ $Ag_2S$ NM/ bulk $Ag_2S$ | 8-12 mg/kg | 180 d | $Ag_2S$ inhibited ammonium oxidizing bacteria, but less than not sulfided AgNPs and $AgNO_3$. | Schlich et al. (2018) |
| Soil microbial community | Natural soil + sludge | PVP-coated (39 nm)/ $AgNO_3$ | 3 to 706 mg/kg (AgNP)<br>138 mg/kg ($AgNO_3$) | 70 d | Sulfided AgNPs caused a significant inhibition on dehydrogenase and nitrification activities, which is accentuated in time. | Samarajeewa et al. (2019) |

PVA: polyvinyl alcohol (Aldrich); PVP: polyvinyl pyrrolidone; mPEG: methoxy-polyethylene glycol

## 4. Effects of silver on plants

Uptake and bioaccumulation of elements by terrestrial plants can be a starting point for transfer to other organisms. From an agronomic and health point of view, the negative effects of silver species on cultivated plants or their accumulation in the tissues of edible plants could pose major problems.

Many studies have attempted to assess and understand the effects of silver compounds on plant species by direct contamination of the matrix (Table 2, Fig 2). Endpoints consist of observations of life traits such as reproduction/germination, biomass and morphology as well as measurements of metabolic and gene response changes.

### 4.1. Effect of AgNPs on plants

#### 4.1.1. Experiments in simplified conditions

As in most toxicity assessments, the first works related to the effects of silver were toxicological rather than ecotoxicological studies and performed under simplified conditions (culture medium, blotting paper). Many studies have reported a variety of adverse effects of silver species on plants.

Seed germination does not appear to be directly affected by AgNPs for many plant species (for example, *Cucumis sativus, Lactuca sativa, Cucurbita pepo, Linum usitatissimum, Solanum spp. Tomatoes, Ricinus communis,* and *Raphanus sativus*) (Barrena et al., 2009; El-Temsah and Joner, 2012; Pittol et al., 2017; Qian et al., 2013; Song et al., 2013; Stampoulis et al., 2009; Yasur and Rani, 2013). However, delays in germination have been observed in *Lolium perenne*, *Hordeum vulgare,* and *Oryza sativa* (El-Temsah and Joner, 2012; Thuesombat et al., 2014) in response to various sizes of nanoparticles. Occasionally, indirect effects have been evoked over several generations, such as a reduction of germination in *Arabidopsis thaliana Columbia* (Geisler-Lee et al., 2012). Since these studies differ in the size and coating of the

particles used, their concentration and the method of exposure, it is difficult to explain these different responses, which may also be attributable to differences in the sensitivity of plant species to silver.

Plant biomass may be affected by exposure to AgNPs, leading to a reduction of shoot length (Mirzajani et al., 2013; Nair and Chung, 2015; Song et al., 2013; Stampoulis et al., 2009; Vishwakarma et al., 2017). Some species exposed to AgNPs exhibit reduced root growth compared to controls (Cvjetko et al., 2017; Geisler-Lee et al., 2012; Nair and Chung, 2015; Pittol et al., 2017; Song et al., 2013; Vishwakarma et al., 2017; Zuverza-Mena et al., 2016). Geisler-Lee et al. (2012) observed a tendency of brown coloration at the extremities of roots. With respect to biomass, after several days of exposure, AgNPs are more phytotoxic and induce a greater biomass loss than ionic silver at the same concentrations (El-Temsah and Joner, 2012; Stampoulis et al., 2009). This decrease in growth/biomass is attributable to metabolic disturbance, as outlined below.

Metabolism is affected by silver in many plants because silver species penetrate the cells and accumulate in plant tissues (Kaveh et al., 2013; Nair and Chung, 2014; Song et al., 2013; Stampoulis et al., 2009; Yasur and Rani, 2013). Geisler-Lee et al. (2014) observed gradual penetration of the whole plant by nanoparticles through the roots 17 days after planting. In some plants, AgNPs are able to penetrate the roots without dissolving through the apoplastic pathway, and nanoparticle size does not significantly impact silver accumulation (Geisler-Lee et al., 2012). The review of Yan and Chen (2019) explain that AgNPs can be transported by intercellular spaces and vascular tissue, and that xylem is the most important vehicle of NPs in plants. Once inside the cell, AgNPs may cause mitochondrial membrane perturbation, leading to increased production of reactive oxygen species (ROS) (Nair and Chung, 2015, 2014; Yan and Chen, 2019). Plant contents of carotenoids, phenols, flavonoids, anthocyanins, and proline are enhanced in the presence of AgNPs (Mirzajani et al., 2013; Nair and Chung, 2015, 2014;

Yasur and Rani, 2013). These compounds allow the plant to lessen oxidative damage and are involved in antioxidant responses, ROS removal and metal chelation (Mirzajani et al., 2013; Nair and Chung, 2015, 2014; Yasur and Rani, 2013). Chlorophyll content, which affects photosynthesis and consequently plant growth, is also reduced after exposure to AgNPs (Mirzajani et al., 2013; Nair and Chung, 2015, 2014; Qian et al., 2013; Song et al., 2013; Vishwakarma et al., 2017). Moreover, Olchowik et al. (2017) observed changes in chloroplast ultrastructure. Perturbations of photosynthesis can cause an excess of electrons, which generate ROS and damage chloroplast structure (Qian et al., 2013). Transpiration is also negatively affected (Stampoulis et al., 2009), as is total soluble carbohydrate content (Mirzajani et al., 2013). These decreases in chlorophyll and total soluble carbohydrate content indicate changes in resource allocation.

AgNPs may affect the expression levels of genes involved in antioxidant mechanisms, such as the ethylene signaling pathway and acquired systemic resistance (Geisler-Lee et al., 2014; Kaveh et al., 2013; Nair and Chung, 2015, 2014). Inhibition of ethylene signaling is the most likely hypothesis that explains the positive effects on plant growth following low-dose foliar treatment with AgNPs (Sadak, 2019), however this effect is not observed when Ag is in the substrate. A strong increase in antioxidant enzymatic activities has been observed (Barbasz et al., 2016; Jiravova et al., 2016; Song et al., 2013; Yasur and Rani, 2013). In addition some changes at genetic level were observed i.e. in the transcription of genes encoding antioxidant effectors, like aquaporins, which have been implicated in the plant water balance (Qian et al., 2013); sulfur assimilation; and glutathione GSH-biosynthesis (Nair and Chung, 2014). These two last changes are related to sulfur metabolism, which plays a role in chlorophyll synthesis and plant growth, thus explaining the observed decreases in chlorophyll content and biomass. In addition, nanoparticles trapped in the center of plasmodesmata could physically block symplastic transport, thus delaying or stopping intercellular transport and contributing to the inhibition of

plant growth (Geisler-Lee et al., 2012). Also, (Sun et al., 2017) have shown that genes related to the auxin receptors were negatively regulated suggesting a decrease of levels of auxin that can explain partially the reduction of growth. Consistent with these changes, a decrease in nutrient content in plants was observed upon AgNPs exposure (Zuverza-Mena et al., 2016).

In most cases, regardless of the concentration used, the observed effect of AgNPs is attenuated compared to $Ag^+$ ions, as shown by Yasur and Rani (2013), (Barbasz et al., 2016), or (Krajcarová et al., 2017) who observed that nanoparticles were less toxic because they remained in the plant peripheral tissues while silver ions penetrated the heart of plant tissues. However, some studies have shown opposite effect. It was reported a stronger effect of AgNPs than silver ions on biomass and transpiration (Stampoulis et al., 2009). Ag content of plants exposed to $AgNO_3$ was lower than plants exposed to AgNPs (Geisler-Lee et al., 2012) and the transcription of some genes was more affected by AgNPs than $AgNO_3$ (Nair and Chung, 2014; Qian et al., 2013). Nevertheless, these last studies did not differ from the previous ones regarding the tested nanoparticles size, concentrations and exposure modes. The obtained conflicting results are of delicate interpretation.

Other studies have examined the role of AgNPs size in toxicity. Overall, smaller particles appear to be more phytotoxic than larger ones (Cvjetko et al., 2017; Geisler-Lee et al., 2012; Thuesombat et al., 2014; Zaka et al., 2016), which may be related to extended dissolution due to the larger specific surface area of smaller particles as well as greater absorption by organisms (Johnston et al., 2010).

In summary, experiments under simplified conditions have shown that AgNPs may have various negative effects on plants. The molecular, biochemical, physiological and individual parameters affected by AgNPs could be used as biomarkers to better understand the subcellular effects of silver on plants and to design studies investigating these effects under more realistic conditions.

### 4.1.2. Experiments with soil matrices

Studies with sowed soils allowed to assess possible effects of silver species on reproduction/germination under more realistic conditions. Geisler-Lee et al. (2014) observed that the germination rates of *Arabidopsis thaliana* seeds decreased significantly over several generations when regularly irrigated with a solution of AgNPs. They also noted that the vegetative phase was prolonged during exposure to AgNPs, whereas the reproductive phase was shortened/accelerated. Unlike many other species, the germination of *Arabidopsis thaliana* was also affected in liquid media (see paragraph 4.1.1).

Effects of AgNPs supplied directly in the soil matrix on plant growth have also been observed. Extremely high doses of AgNPs (500 and 2000 mg $kg^{-1}$) cause significantly smaller phenotypes in *Arachis hypogea* (Rui et al., 2017), while an important decrease in pod biomass is observed even at 50 mg $kg^{-1}$ AgNPs. Decreases in growth are also observed in *Elymus lanceolatus* and *Trifolium pratense* (two forage plants) during exposure to high concentrations of AgNPs (Velicogna et al., 2016). The IC50 was approximately 45-75 mg $kg^{-1}$ in the most sensitive soil. Interestingly, Rui et al. (2017) reported the accumulation of silver in the seeds of *Arachis hypogea* (peanuts) during exposure to AgNPs. As ground nuts are edible, these results raise questions about the risk incurred by consumption.

Changes in metabolism have also been observed. Geisler-Lee et al. (2014) observed a change in the inorganic absorption of nitrogen nutrients after 9 weeks of exposure to AgNPs, likely due to changes in the expression of genes related to nutrient absorption. Also, a decrease in photosynthesis was observed by Das et al. (2018) in tomato plants while Colman et al. (2013) did not observe any changes in photosynthesis at lower concentrations (100 times less) of AgNPs in several species.

Comparisons of the responses of a range of plant species to a given silver exposure have indicated that the effects of AgNPs vary considerably among plant species depending on their

relative susceptibility to silver species (Colman et al., 2013; El-Temsah and Joner, 2012; Velicogna et al., 2016). Velicogna et al. (2016) indicated a role of soil properties in sensitivity to AgNPs, as more toxic effects of silver were observed in plants in sandy soil. This result stays in agreement with Layet et al. (2019) study, who showed that sandy and loam soils lead to a higher bioavailability of AgNPs for tall fescue compare to clay soil. In soil, no trend between AgNPs size and toxicity was observed. Thus, it is possible that the interactions of nanoparticles and silver ions with a complex matrix and potential silver transformations in contact with the soil are sufficient to alter the size-toxicity dependence observed in a simple liquid medium (El-Temsah and Joner, 2012).

### 4.2. Effect of silver sulfidation on plants

Since Ag is mainly found in sulfide form in terrestrial environments, some studies have assessed the effects of silver sulfides (Fig 2).

Pradas del Real et al. (2016) adopted realistic conditions by contaminating soils with Ag-doped sewage sludge at the entrance of a WWTP. They observed a negative effect of silver sulfides on the root growth of rape and wheat, although the transfer of silver from soil to roots was lower than that observed for non-sulfidized AgNPs. Another important observation was the presence of nanosized metallic mixed sulfides including Ag. These species, which form in SS, may have totally different physicochemical behaviors than metallic silver or $Ag_2S$ NPs. The risk of contamination by plant consumption appears to be low and the negative effects on crop yield remain to be examined. Similar results showing limited transfer of silver sulfides in plants were reported by Pradas del Real et al. (2017). The authors found that uptake of silver in wheat roots and its translocation in shoots were more important for AgNPs and ionic silver than $Ag_2S$-NPs. More importantly, they observed that Ag speciation was modified by accumulation in shoots and that a minor part of $Ag_2S$ could be transformed despite its low solubility, probably

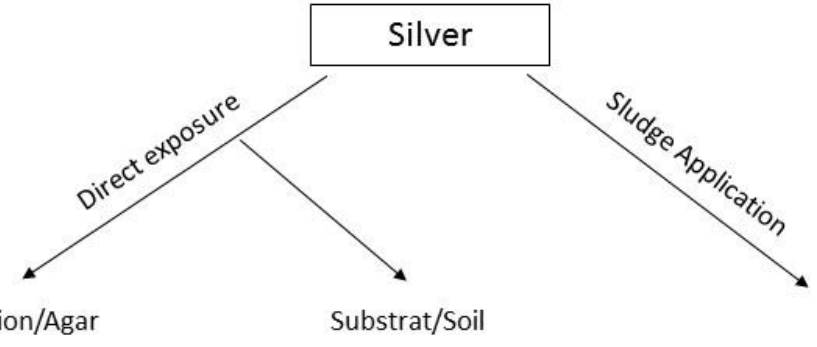
due to rhizospheric activity. Lower transfer of $Ag_2S$-NPs to roots compared to AgNPs was also observed in alfalfa (Stegemeier et al., 2015). The transfer of intact $Ag_2S$-NPs from solution to plant tissues was observed by Wang et al. (2015), without dissolution. Doolette et al. (2015) observed poor transfer of $Ag_2S$-NPs in plants. However, the addition of fertilizers (ammonium thiosulfate and potassium chloride) can increase silver bioavailability for lettuce in soil without biosolids. In the presence of ammonium thiosulfate, silver interacts with thiosulfate to form $Ag(S_2O_3)^-$, which is suspected to be more bioavailable than AgNPs or $Ag_2S$ NPs. KCl also affects the bioavailability of AgNPs and $Ag^+$ (probably due to the formation of soluble Ag-Cl species) but does not affect the bioavailability of $Ag_2S$.

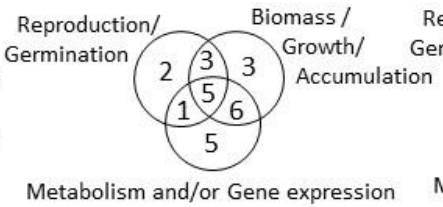
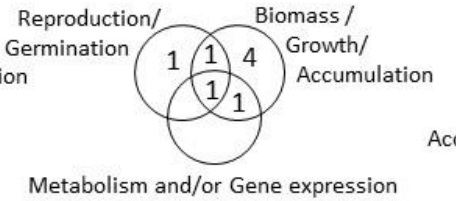
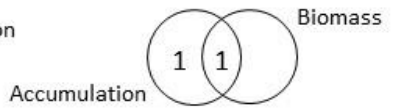
$Ag_2S$-NPs also cause phytotoxicity, and several stress markers have been identified in wheat (Pradas del Real et al., 2017) based on the overexpression of genes with key roles in oxidative stress response, namely CAT (encoding the enzyme catalase) and SOD (encoding superoxide dismutase). Similar effects of exposure to AgNPs had already been demonstrated in other species (Kaveh et al., 2013; Nair and Chung, 2015). Overexpression of metallothioneins, proteins related to metal homeostasis and the trapping of ROS, has also been reported (Pradas del Real et al., 2017). Silicate aggregates could be identified in the cytoplasm of root cells of alfalfa exposed to $Ag_2S$-NPs (Stegemeier et al., 2015), which may reflect the induction of a known plant defense mechanism for the control of metals and pests (Ma and Yamaji, 2006; Wu et al., 2013). Also, P. Wang et al. (2017) showed that $Ag_2S$-NPs can upregulate genes involved in ethylene signaling pathway in wheat and cucumber. Ethylene is linked to the plant senescence and is also a stress hormone.

Globally, silver species trigger physiological stress in plants, resulting in a decrease of growth, especially roots, and consequent effects on yields. Silver ions can easily enter plant tissues, but silver sulfides are less bioavailable and therefore less toxic to plants. More realistic studies are needed to improve knowledge on this topic.

### 4.3. Overview of the effects on plants

Thus, all these studies have shown that silver is toxic for plants. Whatever the concentration, the particle size, the coating, the matrix or the plant species, adverse effects were observed on growth, reproduction and metabolism and stress markers were highlighted. In contrast to what has been seen in microorganisms, in plants, the toxicity seems to increase with the exposure dose. There is no clear impact of the particle size on the Ag toxicity, although one observation of a higher toxicity of very small nanoparticles has been made. In general, despite some studies showing contrasting results, it seems that $AgNO_3$ is more toxic than AgNPs, which itself is more toxic than $Ag_2S$. However, the study of silver sulfides is still growing and first results demonstrate phytotoxicity and accumulation capacity, justifying further works using realistic concentrations to study the effects on crop production and trophic transfer.




| | Solution/Agar | Substrat/Soil | Soil |
|---|---|---|---|
| **Number of studies** | 25 | 8 | 2 |
| **Concentration range** | 0.001 - 10000 mg.$L^{-1}$ | 0.0015 - 5000 mg.$kg^{-1}$ | 0.14 - 400 mg.$kg^{-1}$ |
| **Duration range** | 24h – 10 weeks | 8 days – 72 weeks | 28 - 50 days |
| **Repartition of studies depending on the measured parameters** |  |  |  |



| | | Response Type | Intensity of effects | Response Type | Intensity of effects | Response Type | Intensity of effects |
|---|---|---|---|---|---|---|---|
| **Summary of main results** | Growth | ↘ or = ↗ at low dose | AgNP ≥ or ≤ Ag+ | ↘ or = (but slow) | AgNP < Ag+ | ↘ | AgNP ≥ Ag+ |
| | Reproduction | ↘ or = | AgNP ≥ Ag+ | ↘ | | | |
| | Metabolism/ Genic expression | changes | AgNP ≥ or ≤ Ag+ | changes | | | |

Figure 2: Schematic overview of available studies on AgNP effects on plants

*Table 2: Overview of key studies focusing on the effects of AgNPs on plants*

| Test organism | Matrix | Type of AgNP (size)/control | Concentration/Dose | Time | Main Effects | Reference |
|---|---|---|---|---|---|---|
| *C. sativus*<br>*L. sativa* | Solution | AgNPs (2 nm) | 0.062, 0.1 and 0.116 mg/L | 7 d | No effect on seed germination | Barrena et al. (2009) |
| *A. thaliana* | Solution | Citrate-coated (20, 40, 80 nm)/$AgNO_3$ | 66.84 to 534.72 mg/L (AgNPs)<br>2.6 to 534 mg/L ($AgNO_3$) | 2-4 w | Ag caused a decrease in root length. The content of Ag in plants exposed to $AgNO_3$ was lower than that in plants exposed to AgNP. Toxicity was dependent on the size and concentration of AgNPs. | Geisler-Lee et al. (2012) |
| *A. thaliana* | Solution | PVP-coated (20 nm)/$AgNO_3$ | 1, 2.5, 5, 10, 20 mg/L | 10 d | Ag caused up- and downregulation of many genes. Differences were form-specific. | Kaveh et al. (2013) |
| *C. pepo* | Solution | AgNPs (100 nm)/$AgNO_3$ | 1000 mg/L (AgNPs)<br>1, 10, 100, 1000 mg/L ($AgNO_3$) | 17 d | Biomass and transpiration decreased in the presence of AgNPs, which released few ions; thus, the nanoparticles themselves caused toxicity. Seed germination was not affected. | Stampoulis et al. (2009) |
| *L. esculentum* | Solution | Citrate-coated (10-15 nm) | 0, 50, 100, 500, 1000 and 5000 mg/L | 2-5 w | AgNPs caused a decrease in root elongation but did not affect seed germination. They also caused a decrease in chlorophyll content and fruit productivity and an increase of superoxide dismutase activity. | Song et al. (2013) |
| *R. communis* | Solution | PVP-coated (< 100 nm)/$AgNO_3$ | 0, 100, 200, 500, 1000, 2000 and 4000 mg/L | 7 d | $AgNO_3$ but not AgNPs caused inhibition of seed germination. Ag, in both forms, was absorbed in tissues and caused an increase in ROS enzyme activities and phenolic content. | Yasur and Rani (2013) |
| *A. thaliana* | Solution | AgNPs (20 nm)/$AgNO_3$ | 0.2, 0.5, 1 mg/L | 14 d | AgNPs were more phytotoxic than $AgNO_3$. Ag caused a differential modulation of genes involved in sulfur assimilation and GSH biosynthesis. | Nair and Chung (2014) |
| *O. sativa* | Solution | AgNPs (20; 30-60; 70-120; 150nm)/ $AgNO_3$ | 0.1, 1, 10, 100, 1000 mg/L | 21 d | AgNPs affected seed germination and seedling growth rather strongly depending on the size and concentration of Ag. These effects were due to penetration and transport of Ag through plant tissues. | Thuesombat et al. (2014) |
| *T. aestivum* | Solution | AgNPs (17 ± 5 nm)/$AgNO_3$ | 0, 20, 40, 60 ppm | 24 h | Ag caused cell deformations, oxidative stress and an increase in the production of non-enzymatic antioxidants. | Barbasz et al. (2016) |
| *S. lycopersicum,*<br>*S. chmielewskii*<br>*S. habrochaites* | Solution | AgNPs (27 nm)/ $AgNO_3$ | 50 mg/L | 10 w | AgNPs caused oxidative stress.<br>There was no change in viability. | Jiravova et al. (2016) |
| *E. sativa* | Solution | AgNPs (18 nm)/$AgNO_3$ | 1nM | 6 w | AgNPs stimulated plant growth at certain exposure time points. They induced oxidative stress. | Zaka et al. (2016) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *R. sativus* | Solution | AgNPs (1-10 nm) | 0, 125, 250 and 500 mg/L | 5 d | AgNPs affected growth (diminution of root length), nutrient content (less Ca, Mg, B, Cu, Mn, Zn) and macromolecule conformation (lipids, proteins, lignin, pectin, cellulose). | Zuverza-Mena et al. (2016) |
| *A. cepa* | Solution | Citrate (bigger), PVP & CTAB (smaller)-coated/$AgNO_3$ | 25, 50, 75 and 100 µM | 72 h | $AgNO_3$ was more toxic than AgNPs. At high concentrations, AgNPs caused oxidative stress. The toxicity depended on the characteristics of nanoparticles: size, surface charge and surface coating. | Cvjetko et al. (2017) |
| *V. faba* | Solution | TA-coated (21.7 ± 2.3 nm)/$AgNO_3$ | 10 µmol/L | 7 d | AgNPs did not penetrate in plant tissues, unlike $Ag^+$ ions. | Krajcarová et al. (2017) |
| *A. thaliana* | Solution | PVP-coated (20nm) /$AgNO_3$ | 10 to 150 mg/L (AgNPs)<br>0.12 mg/L ($AgNO_3$) | 3 d | Ag is accumulated. It inhibited the root gravitropism and reduced the accumulation of auxin and the expression of genes involved in auxin receptors. | Sun et al. (2017) |
| *T. aestivum* | Solution | AgNPs /$Ag_2S$-NPs /$AgNO_3$ | 30 µM | 3 w | Ag accumulated in plants and impacted genes involved in oxidative stress, defense against pathogens and metal homeostasis. | Pradas del Real et al. (2017) |
| *M. sativa* | Solution | PVP-coated (6.3 nm) /$Ag_2S$-NPs (7.8 nm) /$AgNO_3$ | 3 mg/L | 6 d | The 3 forms of Ag accumulated in plants. | Stegemeier et al. (2015) |
| *V. unguiculata*<br>*T. aestivum* | Solution | $Ag_2S$-NPs (85 nm) / AgNPs (17 nm) /$AgNO_3$ | 0 to 20 mg/L (AgNPs)<br>0 to 1.6 mg/L (AgNPs)<br>0 to 0.086 mg/L ($AgNO_3$) | 2 w | $Ag_2S$-NPs is accumulated without dissolution. Silver caused growth decrease. | Wang et al. (2015) |
| *Brassica sp.* | Solution | AgNPs (47 nm) /$AgNO_3$ | 1 to 3 mM | | Ag is accumulated in plants and reduced growth. It decreased total chorophyll and enzymatic activities. | Vishwakarma et al. (2017) |
| *C. sativus*<br>*T. aestivum L.* | Solution | $Ag_2S$-NPs (35-120 nm) /$AgNO_3$ | 10 mg/L (AgNP)<br>0.5 mg/L ($AgNO_3$) | 1 w | $Ag_2S$-NPs of 120nm can be absorbed by roots without dissolution. They upregulated genes involved in ethylene signalling pathway. | P. Wang et al. (2017) |
| *A. cepa*<br>*R. sativus* | Solution and agar | AgNPs (10 nm) | 0.001 to 10000 mg/L | 5-7 d | AgNPs inhibited the root growth of onion and improved that of radish. AgNPs did not affect radish germination. | Pittol et al. (2017) |
| *V. radiata* | Agar | AgNPs (20 nm)/$AgNO_3$ | 0, 5, 10, 20 and 50 mg/L | 21 d | AgNPs affected the growth of plants and caused oxidative stress. | Nair and Chung (2015) |
| *O. sativa* | Agar | AgNPs (18 nm) | Up to 60 mg/L | 21 d | At low doses, AgNPs caused an increase in root branching. The dry weight in rice decreased with AgNPs. NPs decreased the content of different elements such as total soluble carbohydrates and chlorophyll. | Mirzajani et al. (2013) |

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| *A. thaliana* | Agar | AgNPs (10 nm)/$AgNO_3$ | 0.2, 0.5 and 3 mg/L | 2 w | AgNPs were absorbed in plant tissues and altered the transcription of antioxidant and aquaporin genes, affecting the homeostasis of water. AgNPs was more toxic than Ag+. | Qian et al. (2013) |
| *L. usitatissimum*<br>*L. perenne*<br>*H. vulgare* | Filter paper /soil | AgNPs (0.6-2 nm; 5 nm; 20 nm) | 0 to 100 mg/L (solution)/100, 250, 500, 1000, 2000, 5000 mg/kg (d.m soil) | 5-7 d | AgNPs slowed seed germination at low concentrations but did not completely inhibit it. | El-Temsah and Joner (2012) |
| *Q. robur* | Substrate (sphagnum peat + coarse-grained perlite) | AgNPs (5 nm) | 0, 5, 25, 50 ppm | Several months | AgNPs were not toxic; only changes in chloroplasts were observed. At some concentrations, mycorrhization increased. Silver was not effective in protecting oak from powdery mildew infection. | Olchowik et al. (2017) |
| *A. thaliana* | Soil | Citrate-coated (20 nm)/ $AgNO_3$ | 0.075 and 0.3 mg/L (AgNPs)<br>0.004 and 0.017 mg/L ($AgNO_3$) | 9 w | Ag accumulated in the whole plant. In presence of AgNPs, vegetative development was prolonged, and reproductive growth was shortened. The germination rates of offspring decreased over several generations. | Geisler-Lee et al. (2014) |
| *E. lanceolatus*<br>*T. pratense* | Soils | PVP-coated (20 nm)/$AgNO_3$ | 0 to 2000 mg/kg | 21 d | $AgNO_3$ was more toxic than AgNPs. Silver had a negative impact on plant growth. The effect depended on the type of soil and species. | Velicogna et al. (2016) |
| *A. hypogea* | Soil mixed with sand | AgNPs (20 nm) | 50, 500, 2000 mg/kg | 98 d | AgNPs accumulated in edible vegetable tissues, a potential risk for humans. AgNPs reduced plant growth and crop yield. | Rui et al. (2017) |
| *L. esculentum* | Soil | AgNPs (10 nm) | 10 to 50 mg/kg | 72 w | Ag disrupted fruit yield. Oxidative stress and Ag uptake were observed. Photosynthesis and $CO_2$ assimilation efficiency have been severely disrupted. | Das et al. (2018) |
| *L. sativa* | Soil | PVP-coated (10 nm)/ $AgNO_3$/ $Ag_2S$ | 1.2 mg/kg (AgNPs)<br>1.3 mg/kg ($Ag_2S$ and $AgNO_3$) | 60 d | Silver forms were poorly bioavailable for lettuce; however the addition of fertilizers (ammonium thiosulfate and potassium chloride) increased its bioavailability. | Doolette et al. (2015) |
| *F. arundinacea* | 4 natural soils | PVP-coated (50 nm)/ $SIO_2$-coated (50 nm)/ $AgNO_3$ | 0.0015 to 0.15 mg/kg | 8 d | The bioavailability of Ag is highly dependent on the nature of the soil. The roots accumulate much more Ag than the leaves. | Layet et al. (2019) |
| *C. lurida*,<br>*J. effusus*,<br>*L. cardinalis*,<br>*M. vimineum*,<br>*P. virgatum* | Soil with/without sludge | PVP-coated (21 ± 17 nm)/$AgNO_3$ | 0.14 mg/kg (AgNPs)<br>0.56 mg/kg ($AgNO_3$) | 50 d | Ag was more likely to accumulate in plant tissues under $AgNO_3$ conditions. The aboveground plant biomass decreased in response to AgNPs in *M. vimineum*. | Colman et al. (2013) |

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| *T. aestivum*<br>*B. napus* | Soil with or without sludge | PVP-coated (500 – 3000 nm) (aggregates) | 14, 18 and 400 mg/kg | 4 w | The transfer of Ag in soil pore water and in plants was low because Ag was present in sulfur form in sludge. However, soil components, microorganisms and plant exudates can induce interconversion between $Ag_2S$ and Ag and increase availability. | Pradas del Real et al. (2016) |

PVP: polyvinyl pyrrolidone, CTAB: cetyltrimethylammonium bromide, TA: tannic acid

## 5. Effects of silver on animals (soil invertebrates)

Studies of soil mesofauna have focused mainly on nematodes, such as the model species *Caenorhabditis elegans*, and earthworms (Table 3). Earthworms, so-called "soil engineers", are commonly used in soil ecology and ecotoxicology experiments because they represent the soil biomass and participate in various important functions such as decomposition and burial of organic matter and other components. A study has also reported that $Ag_2S$ brought *via* the SS tended to remain on the surface after spreading because it is complexed in organic matter. The rain did not induce significant migration to the deeper soil layers, however the activity of the earthworms favored the dispersion of Ag in the soil (Baccaro et al., 2019).

More recently, research has included other groups that may be affected by silver accumulation in plants, such as isopods and collembola. Isopods are frequently used to evaluate the bioavailability of metals due to their accumulation capacity (Kampe et al., 2018; Tourinho et al., 2016), and collembola is another important group of decomposers (McKee et al., 2017).

### 5.1. Effect of AgNPs on soil invertebrates

#### 5.1.1. Experiments in simplified conditions

The nematode *Caenorhabditis elegans*, which is widely used in biology laboratories, has been employed to study the impact of AgNPs on filter paper, water biofilms or culture medium (Fig. 3). Conflicting results on the impact of AgNPs on nematodes have been reported. For instance, low doses (< 1.5 mg $L^{-1}$) of AgNPs did not have any negative impact on survival (Maurer et al., 2016; Roh et al., 2009). Kim et al. (2012) observed an influence of exposure to low concentrations of AgNPs, but aggregation of particles seemed to inhibit this effect. At concentrations of up to 10 mg $L^{-1}$, Kim et al. (2012) observed a dose-dependent effect on survival, but at higher concentrations (above 100 mg $L^{-1}$), the response was not dose-dependent due to particle aggregation.

Another parameter that is strongly affected by AgNPs exposure is reproduction. Inhibition of *C. elegans* reproduction follows a dose-dependent relationship reflecting the modification of the transcription of genes related to reproductive behavior (Kim et al., 2012; Luo et al., 2016; Roh et al., 2009). Luo et al. (2016) also showed that AgNPs toxicity affects subsequent generations by causing germ cell degradation. Interestingly, growth in culture medium is not affected by AgNPs contamination (Roh et al., 2009), and accumulation of silver in nematode tissues was observed (Luo et al., 2016), suggesting that AgNPs could be transferred to higher trophic levels.

Some subcellular stress markers are affected by exposure to AgNPs. For instance, changes have been observed in the production of ROS (Lim et al., 2012), the activity of enzymes related to antioxidative response and mechanisms of defense (Lim et al., 2012), and the transcription of genes related to reproductive behavior (Roh et al., 2009). This damage can even ultimately inhibit the mobility function of nematodes (Yang et al., 2017). Moreover, epidermal wounds likely to facilitate secondary infections were observed in *C. elegans* (Kim et al., 2012), suggesting an integumentary mode of transfer of silver to the animal and an explanation of the observed mortality. *C elegans* is a well-documented model species, but exposure cannot be performed in a more realistic medium like soil.

### 5.1.2. Experiments with soil matrices

Another taxon, annelids, has been studied in more realistic media, namely artificial and natural soils (Fig. 3). Like nematodes, no mortality was observed in earthworms (*Eisenia fetida, Enchytraeus albidus*, *Enchytraeus crypticus*, and *Lumbricus rubellus*) exposed to AgNPs (Bicho et al., 2016; Curieses Silvana et al., 2017; Diez-Ortiz et al., 2015b; Gomes et al., 2013; Makama et al., 2016; Shoults-Wilson et al., 2011), although two studies have shown contrasting effects in *Eisenia fetida* and *Allolobophora chlorotica* (Brami et al., 2017; Garcia-Velasco et al., 2016). *A. chlorotica* is more sensitive to metal contamination than other species (Spurgeon

and Hopkin, 1996). Mortality was observed in *E. fetida* under very high concentration (500 mg $kg^{-1}$) and with small AgNPs (5 nm). Other studies in *E. fetida* did not test very small nanoparticles at such high concentrations. These results further suggest that the size of the nanoparticles likely influences the toxicity of AgNPs. Also, AgNPs appear to be less toxic than $AgNO_3$, as silver ions cause greater mortality (Brami et al., 2017; Diez-Ortiz et al., 2015b; Gomes et al., 2013; Heckmann et al., 2011). Schlich et al. (2013) estimated that ionic silver was twice as toxic as AgNPs for *Eisenia andrei*. Baccaro et al. (2018) showed that AgNPs was less bioaccumulated in earthworms than $AgNO_3$. Similar effects were observed in *E. fetida* in a study by (Diez-Ortiz et al., 2015b).

Like nematodes, earthworm growth is not affected by AgNPs contamination (Curieses Silvana et al., 2017; Shoults-Wilson et al., 2010). However, many negative effects have been reported in annelids. First, reproduction, like in nematodes, is greatly decreased, depending on the concentration (Bicho et al., 2016; Diez-Ortiz et al., 2015b; Gomes et al., 2013; Heckmann et al., 2011; Jesmer et al., 2017; Makama et al., 2016; Novo et al., 2015), with a reduction of cocoon production or a reduction of the number of cocoons/juveniles reaching maturity (Schlich et al., 2013).

Avoidance behavior is also observed during exposure to AgNPs or $AgNO_3$ (Brami et al., 2017; Shoults-Wilson et al., 2011) more pronounced for $AgNO_3$ than for AgNPs (Mariyadas et al., 2018). Silver accumulates in annelid animal tissues (Bourdineaud et al., 2019; Brami et al., 2017; Garcia-Velasco et al., 2016; Hayashi et al., 2013; Makama et al., 2016; Shoults-Wilson et al., 2010; Vittori Antisari et al., 2016) but without biomagnification (Diez-Ortiz et al., 2015b). Stress markers have been detected, like oxidative stress response effectors and stimulation of defense mechanisms (Gomes et al., 2015). Similarly, AgNPs exposure modifies the transcription of genes related to nuclear signaling, transport pathways, reproductive behavior, endocytosis and general defenses (Bourdineaud et al., 2019; Curieses Silvana et al.,

2017; Garcia-Velasco et al., 2017; Hayashi et al., 2013; Novo et al., 2015; Shoults-Wilson et al., 2010). These changes in enzymatic activities and transcription are less elevated than in the presence of ionic silver (Gomes et al., 2015; Novo et al., 2015), in contrast to the greater toxicity of silver ions observed in most experiments. This discrepancy has not been explained. Finally, some studies of other soil invertebrates taxa have shown effects similar to those observed in nematodes and annelids. For instance, reproductive function is affected for collembola (McKee et al., 2017), albeit to a lesser extent than in earthworms according to Velicogna et al. (2016). Similar to nematodes, accumulation of silver is observed in isopods (Tourinho et al., 2016), suggesting possible upward transfer in the trophic chain.

The discrepancies in described results among studies are not surprising because of the very wide range of types of AgNPs that can be used. The size and coating used to stabilize the nanoparticle against dissolution and aggregation can influence the properties of AgNPs and affect their toxicity. For example, the avoidance responses of *Eisenia fetida* exposed to two types of AgNPs of similar size with different coatings (polyvinylpyrrolidone versus oleic acid) differ (Shoults-Wilson et al., 2011). Luo et al. (2016) also observed an effect of nanoparticle size on toxicity, with easier transfer of small particles into the food chain (from *Escherichia coli* to *Caenorhabditis elegans*). In another study, 5 nm AgNPs coated with arabic gum were approximately 9 times more toxic than 8 nm AgNPs coated with polyvinylpyrrolidone, which in turn were approximately 3 times more toxic than 7 nm AgNPs coated with citrate (Yang et al., 2012), suggesting that the difference in toxicity of nanoparticles is partially related to surface coating. Whitley et al. (2013) showed that the coating has effects on aggregation state and partitioning to pore water in soil, which can affect bioavailability and therefore toxicity. By contrast, when the toxicity of two different nanoparticles with the same coating but sizes of 8 and 38 nm was studied, no link between particle size and the amount of $Ag^+$ ions released was found. However, a link between dissolved silver and toxicity was observed. The nature of the

used substrate can significantly affect the toxicity of the metal. A matrix rich in organic matter allows the Ag to complex with humic acids making Ag less bioavailable and therefore less toxic to the organisms (McKee et al., 2019).

### 5.2. Effect of silver sulfidation on soil invertebrates

#### 5.2.1. Experiments with simple matrix

In lethality and growth assays, a decrease in silver toxicity was observed when *Caenorhabditis elegans* was exposed to $Ag_2S$-NPs rather than AgNPs (Levard et al., 2013), even when sulfidation was partial. This decrease in toxicity was attributed to the poorly soluble $Ag_2S$ acting as a passivation layer on the surface of the AgNPs. Starnes et al. (2015) showed that reproduction was the most sensitive parameter to $Ag_2S$. Briefly, Starnes et al. (2015) showed that the low toxicity of sulfidized silver on *C. elegans* was related not only to the decrease in solubility but also to a decrease in the absorption of intact particles. A similar decrease in reproduction was observed by Schultz et al. (2016) who also showed that multigenerational exposure increased the time to first egg laying.

Starnes et al. (2016) also observed effects on gene expression in *C. elegans*. At concentrations corresponding to the EC30, the transcriptomic profiles differed among exposure to $AgNO_3$, silver micro- and nanoparticles (Ag-MNPs) and sulfided silver micro- and nanoparticles ($Ag_2S$-MNPs). Only 11% of the differentially expressed genes were shared among the three exposures. The authors suggested that Ag-MNP toxicity can be partially explained by the release of $Ag^+$ ions and by specific effects of the particles. Regardless of the ions released and their accumulation, cuticle damage seems to be the main mechanism of toxicity of $Ag_2S$-MNPs in *C. elegans.*

Collin et al. (2016) observed that the composition of the matrix, including natural organic matter and inorganic composition, impacts $Ag_2S$-NP toxicity (mortality rate) in *Caenorhabditis*

*elegans*. Thus, studies with simple matrices (solution and agar) can lead to misleading estimations of silver ecotoxicity.

### 5.2.2. Experiments with soil matrices

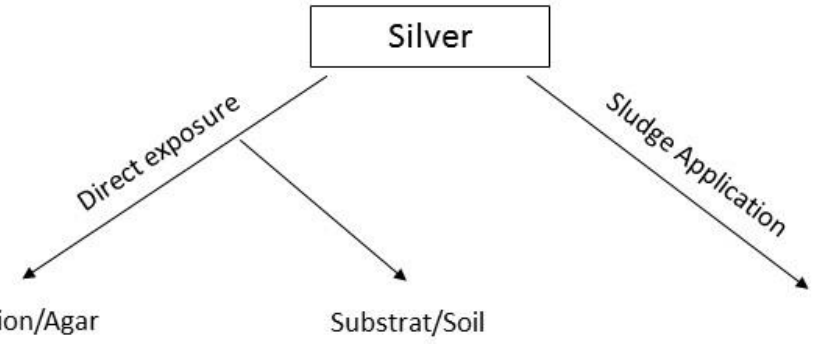
Experiments evaluating $Ag_2S$ toxicity in soil matrices are scarse. Kampe et al. (2018) exposed the woodlouse *Porcellio scaber* to SS with a low $Ag_2S$ concentration. Silver was bioavailable and accumulated in organisms, and a consecutive depuration phase was not efficient enough to eliminate all of accumulated Ag. Silver sulfide is bioavailable to earthworms (Velicogna et al., 2017). Lahive et al. (2017) showed that high doses of AgNPs transformed in wastewater treatment and then aged in natural soil were more bioavailable for earthworms and had greater effects on their survival and reproduction than exposures to metal salt spiked soils. However, at low doses (more realistic), greater internal accumulation of metal ions than $Ag_2S$ occurred, and earthworm survival and reproduction were not affected.

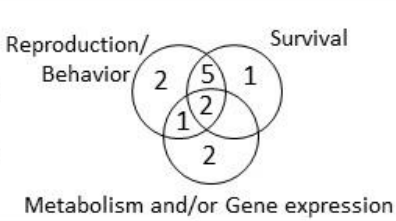
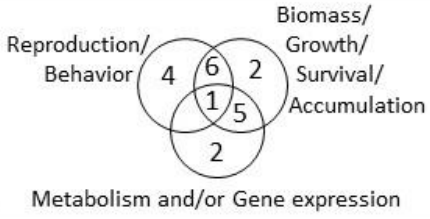
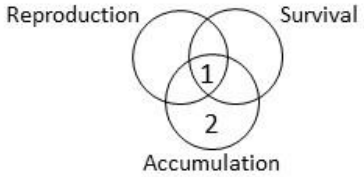
Thus, all forms of silver can accumulate in soil invertebrates, even at low doses. Silver sulfides can significantly alter reproduction functions and survival. However, at realistic doses, silver appears to be less toxic to fauna when it is transformed to $Ag_2S$.

## 5.3. Overview of the effects on soil invertebrates

Thus, for soil invertebrates, all studies have shown that silver exerts a toxicity. Except for exposure to extreme concentrations, survival is rarely affected but a deleterious effect on reproduction is often observed. Metabolism, gene transcription, and behavior undergo changes. Silver, even for the least bioavailable species $Ag_2S$, may be considerably accumulated in soil invertebrates. The study of soil invertebrates also shows that $Ag_2S$ seems less toxic than AgNPs and $AgNO_3$. The particle size seems to impact the toxicity, the smallest nanoparticles being more toxic. In addition, Ag toxicity seems to increase with the concentration, but the aggregation phenomenon which takes place when concentrations are very high decrease the

toxicity. For instance, $Ag_2S$ is significantly bioaccumulated in invertebrates living in soil containing 4 mg $kg^{-1}$ of Ag. More realistic experiments with Ag brought via fermented SS and designed to get final concentration of Ag below 4 mg $kg^{-1}$ in soil are needed.




| | Solution/Agar | Substrat/Soil | Soil |
|---|---|---|---|
| **Number of studies** | 13 | 20 | 3 |
| **Concentration range** | 0.05 - 1000 mg.$L^{-1}$ | 0.0003 - 4400 mg.$kg^{-1}$ | 4 - 78 mg.$kg^{-1}$ |
| **Duration range** | 24 h – 56 days | 1 day – 52 weeks | 14 - 28 days |
| **Repartition of studies depending on the measured parameters** |   |   |   |

| | | Response Type | Intensity of effects | Response Type | Intensity of effects | Response Type | Intensity of effects |
|---|---|---|---|---|---|---|---|
| **Summary of main results** | Survival | ↘ or = | AgNP < Ag+ | ↘ or = | AgNP < Ag+ | ↘ or = | $Ag_2S$ < AgNP < Ag+ |
| | Reproduction | ↘ | AgNP < Ag+ | ↘ or = | AgNP ≤ or ≥ Ag+ | ↘ | |
| | Metabolism/ Genic expression | changes | | changes | | changes | |

Figure 3: Schematic overview of available studies on AgNP effects on soil invertebrates

*Table 3: Overview of key studies focusing on the effects of AgNPs on soil invertebrates*

| Test organism | Matrix | Type of AgNPs (size)/control | Concentration/Dose | Time | Main Effects | Reference |
|---|---|---|---|---|---|---|
| *C. elegans* | Solution | AgNPs (14-20 nm)/ $AgNO_3$ | 0.05, 0.1 and 0.5 mg/L | 24-72 h | The reproduction potential and the expression of the sod-3 and daf-12 genes decreased at the two highest concentrations. | Roh et al. (2009) |
| *C. elegans* | Solution | Citrate- (7 nm), PVP- (8 nm), PVP- (38 nm), GA- (5 nm and 22 nm) coated/ $AgNO_3$ | 20 to 46 µM | 3 d | There was a linear correlation between AgNPs toxicity (for the growth parameter) and dissolved silver but no correlation between size and toxicity. Coating influenced the toxicity. A lower ionic strength medium resulted in greater toxicity. | Yang et al. (2012) |
| *C. elegans* | Solution | Citrate-coated (25 ± 9 nm)/ $AgNO_3$ | 0 to 1.5 mg/L (AgNPs)<br>0 to 0.1 mg/L ($AgNO_3$) | 24-48 h | The toxicity of AgNPs on growth is linked with the endocytosis of the nanoparticles. Endosome formation could be necessary for inducing toxicity in vivo. No significant effect on viability was observed. | Maurer et al. (2016) |
| *C. elegans* | Solution | Citrate or PVP-coated (23, 26, 70, 83 nm) | 0.01, 0.1, 0.5, 1, 50, 20 mg/L | 65 h | The inhibition of locomotion of *C. elegans* depends on surface properties, diameter and exposure time. | Yang et al. (2017) |
| *C. elegans* | Solutions | PVP-coated/$AgNO_3$ | 0.05 to 4.5 mg/L (AgNPs)<br>0.25 to 10 mg/L ($Ag_2S$)<br>0.0025 to 0.085 mg/L ($AgNO_3$) | 24 h | Sulfidized AgNPs were less toxic than AgNPs in terms of the reproduction parameter. Reproduction is a more sensitive parameter than mortality or growth. | Starnes et al. (2015) |
| *C. elegans* | Solutions | PVP-coated/$AgNO_3$ | 350 mg/L (AgNPs)<br>1500 mg/L ($Ag_2S$)<br>10 mg/L ($AgNO_3$) | 48 h | The mechanisms of toxicity of the 3 forms of Ag differed because only 11% of the differentially expressed genes were common for the 3 types of exposure. | Starnes et al. (2016) |
| *E. fetida* | Solution/ OECD soil | PVP AgNPs (5 ± 2 nm) | 0 to 500 mg/kg | 14-56 d | AgNPs in solution were absorbed by the derma and caused accumulation of Ag, loss of mass and mortality. Soil AgNPs exposure caused accumulation mainly in the gut epithelium; the AgNPs exposure pathway was ingestion of contaminated soil and not integumental. | Garcia-Velasco et al. (2016) |
| *C. elegans* | Agar | Citrate-coated (<50 nm)/ $AgNO_3$ | Up to 1000 mg/L | 24-48 h | AgNPs negatively affected biological surfaces of *C. elegans* (severe epidemic edema and burst), survival and reproduction; these effects decreased at higher concentrations due to the aggregation of nanoparticles. | Kim et al. (2012) |
| *C. elegans* | Agar | AgNPs (20 - 30 nm)/ $AgNO_3$ | 0.1, 0.5 and 1 mg/L | 24 h | NPs caused decreases in ROS formation, expression of PMK-1, p38 MAPK, and hypoxia-inducible factor, GST enzyme activity, and reproductive potential in wild type, unlike the pmk-1 mutant. | Lim et al. (2012) |

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| *C. elegans* | Agar | AgNPs (58.3 ± 12.9 nm)/ $AgNO_3$/ $Ag_2S$ (64.5 ± 19.4 nm) | 0.75 to 24 mg/L (AgNPs)<br>7.5 to 240 mg/L ($Ag_2S$)<br>0.05 to 1.52 mg/L ($AgNO_3$) | F0-F10 generation | The 3 forms of silver mostly affected reproduction and body length during multigenerational exposure. | Schultz et al. (2016) |
| *C. elegans* | Culture plates | PVP-coated (25 and 75 nm) (accumulated in *E. coli* and transferred to *C. elegans* by alimentation) | 0, 1, 5, 25 mg/L | F0-F5 generation | AgNPs was accumulated in gut lumen, subcutaneous tissue and gonad. AgNPs affected the reproduction and life span. AgNPs could also affect the next generation: some Ag was present in the F1 generation, and there was an increase in germ cell death in the F2 and F3 generations. | Luo et al. (2016) |
| *C. elegans* | Culture plates | PVP-coated (51 ± 12 nm)/$AgNO_3$ | 0 to 15 mg/L (AgNPs)<br>0 to 0.02 mg/L ($AgNO_3$) | 24 h | The increased sulfidation degree of AgNPs reduced the mortality and growth rates observed in *C. elegans*. | Levard et al. (2013) |
| *C. elegans* | Culture plates | PVP-coated (51 ± 12 nm)/$AgNO_3$ | 0 to 15 mg/L (AgNPs)<br>0 to 0.02 mg/L ($AgNO_3$) | 24 h | The mortality of *C. elegans* due to sulfidized AgNPs is influenced by the inorganic and organic composition of the media. At equivalent concentrations, $AgNO_3$ is more toxic. | Collin et al. (2016) |
| *E. fetida* | Artificial soil | PVP-coated (56 nm) and OA-coated, (50 nm)/ $AgNO_3$ | 10, 100 and 1000 mg/kg | 28 d | Ag had no significant effect on the growth and viability of *E. fetida*, and a decrease in reproduction was observed for both forms of Ag. Accumulation in tissues was greater for $AgNO_3$. No effect of coating was observed. | Shoults-Wilson et al. (2010) |
| *E. albidus* | OECD soil | PVP-coated (30-50 nm)/ $AgNO_3$ | 0 to 1000mg/kg | 6 w | $AgNO_3$ is more toxic than AgNPs for *E. albidus* with regard to survival, reproduction and the expression of selected genes. The response depends on the concentration and the nature of Ag. | Gomes et al. (2013) |
| *E. fetida* | OECD soil | Not coated (10 nm)/ $AgNO_3$ | 0, 100, 300, 600, 900, 1500 mg/kg (AgNPs)<br>0, 25, 50, 75, 100, 200 mg/kg ($AgNO_3$) | 4-28 d | $AgNO_3$ caused mortality at the highest concentrations. Defense mechanisms against Ag involved total glutathione, glutathione peroxidase and glutathione reductase but were not enough to prevent oxidative damage. | Gomes et al. (2015) |
| *E. fetida* | OECD soil | PVP-coated (5 ± 2 nm)/$AgNO_3$ | 0, 0.05 and 50 mg/kg | 1, 3, 14 d | No effects on mortality and biomass were observed. Both forms of Ag caused changes in the metal detoxification mechanism (metallothionein gene and protein) and antioxidant response system (catalase gene and protein). | Curieses Silvana et al. (2017) |
| *E. fetida* | OECD soil | PVP-coated (60 nm)/ $AgNO_3$ | 2, 10, 50 mg/kg | 10 d | Both forms of Ag were bioaccumulated (2-15 times more for the ions) and caused oxidative stress. AgNPs suppressed the expression of genes involved in general defense unlike ions. | Bourdineaud et al. (2019) |

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|---|---|---|---|---|---|---|
| *L. rubellus* | Artificial soil + manure | PVP-coated (1-10 nm)/ $AgNO_3$ | 500 mg/kg | 5 w | Exposure to Ag caused accumulation of Ag in organisms (more with AgNPs than with AgNO3) and a decrease in the unsaturation degree of fatty acids. | Vittori Antisari et al. (2016) |
| *E. fetida* | Ref. soils: OECD and LUFA 2.3 | PVP-coated (5 ± 2 nm) | 0.05 and 50 mg/kg | 3-14 d | AgNPs caused a loss of mass, a decrease of coelomocyte viability, and increases in catalase activity and DNA damage. Effects appeared earlier in LUFA than in OECD. | Garcia-Velasco et al. (2017) |
| *F. candida* | Ref. soils: 01A, LUFA 202, OECD | NM-300K (<20 nm)/$AgNO_3$ | 0.0003 to 30 mg/kg (AgNPs)<br>12.5, 25, 50 mg/kg ($AgNO_3$) | 140 d | Reproduction was affected by AgNPs, even at low concentrations. The nature of the soil influenced the availability of Ag and thus its toxicity. | McKee et al. (2017) |
| *E. fetida* | Natural/ Artificial soil | PVP-coated (10 nm)/ PVP and OA (30-50 nm), citrate (15-25 nm)/ $AgNO_3$ | 0.3 to 54 mg/kg (AgNPs)<br>0.3, 1, 9, 18, 27 mg/kg ($AgNO_3$) | 48 h | Earthworms systematically avoided soils spiked with silver, immediately for $AgNO_3$ and after approximately 48h for AgNPs. This difference could not explained by release of silver ions. No change in the microbial community was observed. | Shoults-Wilson et al. (2011) |
| *E. andrei* | RefeSol01A | NM-300K (15 nm)/$AgNO_3$ | 15 to 200 mg/kg | 56 d | The number of cocoons and juveniles who became adults decreased with Ag. $AgNO_3$ was not more toxic than AgNPs. | Schlich et al. (2013) |
| *E. fetida* | Natural soil | PVP-coated (30-50 nm)/ $AgNO_3$ | 1000 mg/kg | 28 d | $AgNO_3$ (but not AgNPs) significantly affected survival. Total reproductive failure was observed in both treatments. | Heckmann et al. (2011) |
| *E. fetida* | Natural soil | AgNPs (82 nm)/ $AgNO_3$ | 500 mg/kg | 14 d | AgNPs and $AgNO_3$ were bioaccumulated similarly. Two Ag forms caused changes in the expression of genes related to oxidative stress but in a time-shifted manner. | Hayashi et al. (2013) |
| *E. fetida* | Ref. Soil LUFA 2. | Not coated (50 nm)/ $AgNO_3$ | 0 to 4395 mg/kg (AgNPs)<br>0 to 1758 mg/kg ($AgNO_3$) | 1, 9, 30, 52 w | $AgNO_3$ causes mortality and affects reproduction, while AgNPs have an effect on reproduction only. The toxicity of $AgNO_3$ decreases over time, unlike AgNPs. Ag accumulates in worms. | (Diez-Ortiz et al., 2015a) |
| *E. fetida* | Ref. Soil LUFA 2.2 | PVP AgNPs (50 nm)/$AgNO_3$ | 0, 18, 45, 112, 281, 703 and 1758 mg/kg | 28 d | Less than 10% mortality for AgNPs and higher mortality for $AgNO_3$. Both forms affected reproduction. The incorporation of the two forms of Ag is driven by different exposure routes and toxicokinetic mechanisms, but the final toxicodynamic responses are the same. | Novo et al. (2015) |
| *P. pruinosus* | Ref. Soil LUFA 2.2 | Alkane-coated (3-8 nm)/ $AgNO_3$ | 30, 60 mg/kg and 10, 20 mg/L for soil and food exposure, respectively | 21 d | *P. pruinosus* easily accumulated Ag in a storage compartment (hepatopancreas) in its tissues by food or soil exposure. Biomagnification of Ag is possible. | Tourinho et al. (2016) |
| *E. crypticus* | Ref. Soil LUFA 2.2 | NM-300K(17 ± 8 nm, 5 nm)/ $AgNO_3$ | 0, 20, 60, 115, 170 mg/kg (AgNPs)<br>0, 24, 48, 72, 96 mg/kg ($AgNO_3$) | 46 d | Both forms affected hatching, survival and reproduction. AgNPs had an embryotoxic effect, and $AgNO_3$ delayed hatching. | Bicho et al. (2016) |

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| *L. rubellus* | Natural soil | BSA (20 nm), chitosan (35 nm), PVP (50 nm)-coated/ $AgNO_3$ | 0 to 250 mg/kg (AgNPs)<br>1.5 and 15 mg/kg ($AgNO_3$) | 28 d | No clear effect of the size of the particles on their toxicity on reproduction was observed. Coating (and its charge) influenced toxicity. | Makama et al. (2016) |
| *F. candida*<br>*E. andrei* | Natural soil | PVP-coated (20 nm)/ $AgNO_3$ | 0, 4, 10, 25, 60, 145, 347, 833, 2000 mg/kg | 28 or 35 d | The type of soil (silty/ sandy) influenced the toxicity (on survival and reproduction) of Ag. $AgNO_3$ was more toxic than AgNP. *E. andrei* was more sensitive than collembolan. | Velicogna et al. (2016) |
| *A. chlorotica* | Natural soil | Not coated (80 nm)/$AgNO_3$ | 0 to 1000 (AgNPs)<br>0, 12.5, 25, 50, 100 mg/kg ($AgNO_3$) | 14 d | Ag affected survival at higher concentrations. Avoidance behavior was observed. There was a significant difference in biomass between worms exposed to AgNPs and AgNO3. With AgNO3 treatment, more silver accumulated. | Brami et al. (2017) |
| *E. andrei*<br>*F. candida* | Natural soil | PVP-coated (38.6 ± 9.8 nm)/AgNO3 | 9 to 833 mg/kg (*E. andrei*)<br>10 to 833 mg/kg (*F. candida*) | 28 d | AgNPs is more toxic than $AgNO_3$ to *E. andrei*, but there was no difference for *F. candida*. Reproduction was mainly affected. | Jesmer et al. (2017) |
| *P. scaber* | OECD soil + SS | NM-300K (15 nm) | 14 mg/kg | 14 d | Sulfided AgNPs were bioavailable for *P. scaber* and were not totally eliminated after 14 days of depuration. | Kampe et al. (2018) |
| *E. andrei* | Natural soil (+SS) | PVP-coated (20 and 40 nm)/$AgNO_3$ | 4 mg/kg (20 nm AgNPs)<br>78 mg/kg (40 nm AgNPs)<br>1 mg/kg ($AgNO_3$) | 21 d | AgNPs accumulated slightly in tissues of *E. andrei*. | Velicogna et al. (2017) |
| *E. fetida* | Natural soil + SS | PVP-coated (50 nm)/ $AgNO_3$ | 9 to 2200 mg/kg | 14, 28 d | Sulfided AgNPs were bioaccumulated and decreased the earthworm survival and reproduction for the higher concentrations. | Lahive et al. (2017) |

PVP: polyvinyl pyrrolidone; OA: oleic acid; GA: gum arabic; BSA: bovine serum albumin
SS: sewage sludge
sAgNPs: sulfidized AgNP

## 6. Conclusions and areas of future research

In conclusion, studies have shown that direct exposure to AgNPs 1) reduces the activity and abundance and changes the diversity of soil microorganisms, 2) results in silver accumulation in plants and animals, and 3) induces biological effects in microorganisms, plants, and soil invertebrates.

Toxicity mechanisms are still in debate but trends can be drawn. First, the size-dependent toxicity of AgNPs has been often described. However, toxicity experiments with varying AgNPs sizes at equal surface area should be performed rather than those with a given particle concentration with the difficulty of measuring surface area in complex media where aggregation may occur. Yet, just considering intrinsic properties of AgNPs, it has been shown that down to 5 nm and for various coatings, no change in surface energy has been observed (Ma et al., 2012) which may comfort the idea that if a nano-effect exists regarding surface energy and dissolution behavior (for example, increase kinetic of dissolution per surface area unit), it should be observed for particles sizes below 5 nm. At these small sizes, it has been shown that the Trojan-horse mechanisms can be potentially releasing high levels of toxic Ag ions in cells (Hsiao et al., 2015)**.** Then, few studies also observed an effect of nanoparticle coating. On the other hand, what is certain is the influence of substrate composition on toxicity. Effects observed in solution, in artificial soil, in various soils and in soil/sewage sludge mixtures vary a lot. Overall, toxicity for all organisms is more moderate in complex medium rich in clays and organic matter. Toxicity also varies depending on the exposure concentrations. As of 0.01 mg $kg^{-1}$ of silver, the majority of microorganism, plant and animal species were at least slightly affected. The toxicity increases with dose in plants and invertebrates however in microorganisms it is impossible to generalize. The maximum toxicity levels vary greatly from one taxon to another, from one activity to another, and are highly dependent on the overall community present in the environment. Finally, toxicity is different from one studied species to another. Given the wide

range of studies and adopted conditions, reports of contradictory effects are not surprising. The literature suggests that the toxicity of AgNPs is partially related to their dissolution and $Ag^+$ ion release, although it is difficult to estimate the relative contributions of these phenomena. Nanoparticle-specific effects are also observed.

Thus, in the past 20 years, research on AgNPs ecotoxicity has been mainly focused on native metallic AgNPs. More environmentally relevant form of Ag (such as $Ag_2S$) are insufficiently considered even though these are the relevant forms that the living organisms will be mostly exposed to. This is particularly true for soil organisms exposed to Ag through sewage sludge amendment. As a general rule, silver sulfide, the main transformation product of metallic and ionic Ag in sewage sludge, is less toxic than AgNPs and $AgNO_3$ for all organisms. It is indeed more stable in the environment and less bioavailable. Indeed, interactions with microorganisms and root exudates the oxidizing species naturally present in the environment can lead to dissolution of $Ag_2S$ and cause toxicity. In some cases, because of their small sizes, $Ag_2S$ nanoparticles can be bioavailable to plants and animals without dissolution. This partial stability makes the $Ag_2S$ exert a moderate toxicity but on the long term (effect increases after weeks or months of exposure). In addition, low doses, quite realistic or approaching, are enough to affect organisms. Indeed, taking into account the very wide range of silver concentrations observed in sewage sludge (between 1 and 850 mg $kg^{-1}$, the sewage sludge dose allowed for spreading (in the European Union: 30 tons of dry matter per hectare on 10 years - Directive 86/278/ECC, 1986), and the possibility of adding this quantity at once or in several times (max 10 times, with annual spreading), the actual concentrations that should be observed in the field could vary between 0.003 and 25 mg $kg^{-1}$. However, the most realistic concentrations would be 0.12 mg $kg^{-1}$ if we consider that there is 20 mg $kg^{-1}$ of Ag in the SS in average and that it is realistic to spread it every two years. The studies have not gone down to this dose but we already see effects for doses that approach it. Thus, as of 0.14 mg $kg^{-1}$ of silver sulfide (brought by digested sewage

sludge) in soil, a plant species had a lower biomass. As of 0.56 mg $kg^{-1}$ of $Ag_2S$ in soil, decrease of nitrification and other enzyme activities have been observed, with a gradual increase in toxicity over time. Similarly, on soil invertebrates, with 4 mg $kg^{-1}$, we already see bioaccumulation. The impact on the animals goes unnoticed but the bioaccumulation along the trophic chain remains possible and the highest trophic levels could receive high doses. The likely realistic concentration that we calculated already exceeds the minimum concentrations for which effects have been observed in microorganisms and plants.

Until recently, exposure studies did not consider whether the conditions adopted sufficiently reflected actual conditions in the environment, including the matrix, concentration and chemical form of silver, and duration of exposure. Data on sulfided silver added under realistic conditions are missing. Future studies should explore more realistic conditions when assessing the roles of these factors as follows:

*Matrix.* Simplified matrix studies are extremely important and useful for understanding the behavior and effects of a compound and, in turn, the mechanisms of toxicity. Such studies have been essential for understanding the hazard of Ag released into the environment. However, it is now necessary to deepen our knowledge using realistic matrices: (i) direct application of Ag to soil does not reflect its deposition in nature, which mainly occurs *via* the spreading of digested sewage sludge. Spiking sewage sludge with Ag at the end of the WWTP is similarly unrealistic because it neglects potential changes that may occur during the fermentation/digestion phase, which stabilizes and sanitizes the sewage sludge prior to spreading. The maximum and realistic degree of sulfidation is obtained in WWTPs. Exposure to $Ag_2S$ without biosolids and exposure to contaminated biosolids (in which the major form of silver is $Ag_2S$) have not been shown to provide the same bioavailability of Ag to plants (Doolette et al., 2015). Therefore, sewage sludge should be used to mimic natural contamination in the terrestrial environment. Also, (ii) natural soils should be used to estimate the real risk of contaminated sewage sludge spreading.

Experimental designs with soil are often used to study the effects of Ag species on microbial communities but remain scarce with plant and terrestrial invertebrates (Tables 1, 2 and 3 and Fig. 1, 2 and 3).

*Concentration.* The ranges of tested concentrations in previous studies are very broad (and often very elevated) (Fig. 1, 2 and 3). It will be important to assess the effects of silver at environmental concentrations. Such doses would be on the order of mg of Ag per kg of soil or even tens of mg $kg^{-1}$ to mimic the legal possibility of spreading large quantities of sewage sludge at one time (Directive 86/278/ECC, 1986).

*Physico-chemical form of silver.* $Ag_2S$-NPs formed in sewage sludge have different properties than those synthesized in the laboratory. In that sense, the effects of crystallinity, partial sulfidation and formation of mixed metallic sulfides on toxicity should be investigated. $Ag_2S$-NPs forming in the sewage sludge in presence of high concentration of organic matter may be more amorphous than particles formed in the laboratory and exhibit different stability and toxicity. Also depending of the size, shape and aggregation state of the AgNPs that enter in the sewer system, the particles may be only partly sulfided potentially affecting their dissolution behavior. Finally, $Ag_2S$-NPs are more likely to form as mixed sulfides in SS than to be present as pure $Ag_2S$-NPs. Metals with a high affinity for sulfide phases, including Zn and Cu, are present at high concentrations in sewage sludge and may favor the formation of these phases. More fundamental studies are needed to investigate the formation and properties of these mixed sulfides to better predict the fate of silver in the terrestrial environment. Although challenging, the characterization of transformation products is essential to better understand their fate in the terrestrial ecosystems and resulting risks. It relies on the use of a combination of advanced techniques (such as X-ray based synchrotron techniques).

*Exposure duration.* The exposure duration of organisms in ecotoxicological studies is also important for observing long-term effects of silver in the environment rather than solely

immediate effects. Like any contaminant, silver can undergo transformations long after its arrival in the soil. The use of punctual and non-chronic contamination is another point of divergence of studies from reality. Under actual agronomic conditions, contaminated SS may be applied to soil annually, and thus long-time-scale studies with repeated applications should be designed to more fully take into account the risks of AgNPs. Ecotoxicological studies examining natural soil chronically treated with contaminated sewage sludge in which silver at realistic concentrations has had time to undergo transformation to sulfides and with sufficient exposure times are lacking. Only one study has examined the effect of multiple additions of silver over time on microflora (Schlich et al., 2016), and the results showed that the effects differed depending on whether the same amount of silver was applied once or several times. However, in this study, the matrix was contaminated with silver directly and not through sewage sludge.

*Range of shape and size.* The AgNPs studied correspond to homogeneous nanoparticles, most often spherical, created to study their toxicity. Different range of sizes have been studied but the toxicity of one size range at a time is evaluated. However, nanoparticles released from nanofunctionalized products are of various sizes and forms. Gagnon et al. (2019) showed that the particles that were released during the washing of socks containing AgNPs measured between 50 and 400 nm for the majority, with 50 to 75% measuring less than 100 nm. Nevertheless, some particles can reach 800-900 nm. These particles are of various shapes, in leaves, spherical or irregular. The specific surface area of these particles therefore varies enormously depending on their size and shape, which can certainly affect their toxicity, even after sulfidation. Thus, future research would be well-advised to study aged AgNPs and released from nanofunctionalized products, after transformation in a wastewater treatment plant, in order to evaluate what is actually released into the environment. Nowack and Mitrano (2018) have also tried to develop a method to produce these aged AgNPs.

Therefore, more studies using realistic pathways of Ag release into the environment are needed. Firstly, to improve solid but insufficient knowledge of the effects of silver species, and especially $Ag_2S$, on soil ecosystems. Besides, it is important to reconcile studies showing the absence of effects on microbial processes performed by diverse and abundant microbial taxa with others showing the absence of effects on processes performed by less diverse and abundant microbial taxa (such as nitrification). Continued mechanism of action studies focused on tissular and cellular targets of silver using different types of biomarkers conducted in parallel will further clarify these effects. In addition, it will be important to further explore the possible transfer of silver into the food chain, which has yet to be confirmed (question addressed by Luo et al., 2016; Rui et al., 2017; Wu et al., 2018), and to study the effect of silver on crop yields.

Defining a specific regulation for the spreading of sewage sludge on soil is complex and cannot be solely based on total Ag concentration. Sulfidation of Ag and the relatively good stability of $Ag_2S$ in sewage sludge and soil decrease the potential harm of Ag at least in the short term. One can argue that no further research is needed based on these first observations, however important questions remain especially regarding the long-term behavior (bioavailability, accumulation) of these transformed species and their effect on fauna and flora. However, with hindsight, the strong awareness about potential ecotoxicity of silver raised 15 years ago (initially based on studies focusing on the ecotoxicity of the pristine metallic AgNPs) is probably unjustified in regards of its transformation and relatively low predicted release concentrations. For these reasons, although important questions remain, the interest of the scientific community for silver ecotoxicity slowed down.

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**References**

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## 2) Objectifs et démarches de cette thèse

Cette thèse a pour objectifs d'approfondir les connaissances sur les effets de l'Ag et de ses dérivés (sulfures d'Ag) dans l'environnement, comme le recommandait l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) dans son rapport de 2015 sur « l'évaluation des risques sanitaires et environnementaux liés à l'exposition aux nanoparticules d'argent ».

Plus précisément, nous avons évalué :

- la toxicité de l'Ag (sous forme ionique, métallique nanoparticulaire et sulfurée nanoparticulaire) sur divers organismes invertébrés vivant dans ou à la surface du sol : le vers de terre épigé *Eisenia fetida*, l'escargot terrestre *Cantareus aspersus* et le criquet *Locusta migratoria*, de manière plus ou moins approfondie ;
- la toxicité de l'Ag sur la diversité et les activités des microorganismes naturellement présents dans plusieurs types de sol ;
- le risque de transfert de l'Ag dans les réseaux trophiques à partir d'une chaîne trophique plante-animaux.

Pour ceci, nous avons mené à bien 4 expérimentations en microcosmes, où divers organismes, microbiens, animaux et végétaux, étaient exposés à diverses formes d'Ag via des matrices artificielles (« sol » OCDE) ou naturelles (sol agricole ou sol de jardin prélevés dans l'environnement).

La première expérience consistait en l'exposition en microcosmes de groupes de vers de terre *Eisenia fetida* dans un sol artificiel OCDE à de l'argent ionique ($AgNO_3$) et nanoparticulaire (AgNPs NM300K) à une gamme de concentration allant de 33 à 277 mg $kg^{-1}$. La biomasse, la mortalité, la bioaccumulation d'Ag, la spéciation de l'Ag dans le sol et les vers de terre ainsi que la localisation de l'Ag à l'intérieur du vers de terre furent examinés (voir chapitre II).

La seconde expérience consistait en l'exposition en microcosmes de groupes de vers de terre *E. fetida* dans un sol agricole naturel cette fois, prélevé peu de temps avant le lancement de l'expérience, n'ayant pas été congelé ni séché afin de préserver la microflore naturelle de ce sol. Une gamme de concentrations plus réaliste fut appliquée (12, 17, 25 mg $kg^{-1}$ matière sèche (m.s.)). L'exposition a duré 5 semaines. La contamination du sol s'est faite de deux manières :

- directe : ajout de $AgNO_3$ ou AgNPs NM300K directement dans le sol ;
- indirecte : ajout de $AgNO_3$ ou AgNPs NM300K dans des bioréacteurs contenant de la boue d'épuration. Après une fermentation anaérobie de 4 semaines, afin de provoquer les transformations chimiques que subit l'Ag au cours des traitements dans les stations d'épuration, ces boues contaminées furent ensuite ajoutées au sol.

La biomasse, la reproduction, la survie, la bioaccumulation de l'Ag, la spéciation de l'Ag bioaccumulé, ainsi que l'expression de 3 gènes impliqués habituellement dans les mécanismes de défense contre les métaux furent examinés chez les vers de terre. L'effet sur les microorganismes du sol fut surveillé par des mesures de respiration et de composition des communautés microbiennes grâce à des prélèvements ADN. La spéciation de l'Ag dans le sol fut également analysée (voir Chapitre III).

La 3$^{e}$ expérience, fortement inspirée de la 2$^{nde}$, avait pour but de préciser certains résultats obtenus. Elle consistait en l'exposition en microcosmes de groupes de vers de terre *E. fetida* dans deux types de sols : un sol agricole naturel (provenant du même site que précédemment) et un sol de jardin. La contamination a été réalisée selon les deux voies utilisées pour l'expérience 2 : ajout direct d'$AgNO_3$ ou AgNPs NM300K ou ajout indirect de l'argent via de la boue d'épuration digérée anaérobiquement au préalable. Une seule concentration fut appliquée : 10 mg $kg^{-1}$ (m.s.). L'exposition a duré 5 semaines également. La biomasse, la reproduction, la survie, la bioaccumulation de l'Ag et l'expression de plusieurs gènes impliqués dans les mécanismes de défense furent examinés chez les vers de terre, à 3 pas de temps

différents (1, 3 et 5 semaines). Concernant les microorganismes du sol, la nitrification et la dénitrification furent mesurées. Aussi, la composition des communautés microbiennes fut examinée grâce à des prélèvements d'ARN (voir Chapitre III).

Enfin, la 4$^{e}$ expérimentation a été réalisée en 2 temps. Dans un premier temps, diverses espèces végétales (navet, chou, chanvre, laitue, ray-grass, radis, trèfle et blé) ont été exposées à un sol agricole naturel mélangé à de la boue contaminée par de l'Ag avant la digestion anaérobie (concentration unique dans le sol à 10 mg kg$^{-1}$ d'Ag (m. s.)). L'exposition a duré 7 semaines. La bioaccumulation d'Ag par les différentes espèces fut comparée.

Dans un second temps, l'espèce végétale qui avait davantage bioaccumulé l'Ag durant la première étape, le Ray-grass, fut cultivée à plus grande échelle. L'apport d'Ag s'est fait de deux manières comme dans les expériences 2 et 3. Un premier apport direct avec l'ajout d'AgNPs directement dans le sol et un second apport indirect avec l'ajout de boue dopée avec de l'Ag digérée anaérobiquement. Une concentration unique à 10 mg kg$^{-1}$ (m.s.) d'Ag dans le sol naturel agricole (m. s.) fut choisie. Dans ces mésocosmes, des groupes de consommateurs primaires, l'escargot *Cantareus aspersus* et le criquet *Locusta migratoria*, furent ajoutés et exposés 5 et 2 semaines respectivement et de manière simultanée. Dans certains mésocosmes, les animaux avaient accès au sol et aux végétaux. Dans d'autres, les animaux n'avaient accès qu'aux végétaux grâce à un filet étendus à quelques cm au-dessus du sol. Les traits de vie du Ray-grass (croissance) et des animaux (biomasse, survie) furent surveillés. La bioaccumulation de l'Ag fut analysée chez ces 3 espèces (voir Chapitre IV).

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# Chapitre II

# BIOACCUMULATION ET EFFETS TOXICOLOGIQUES DE L'ARGENT CHEZ LE VERS DE TERRE *Eisenia fetida*

La bibliographie montre que l'Ag est bioaccumulé par les organismes du sol (Bourdineaud et al., 2019; Brami et al., 2017; Diez-Ortiz et al., 2015a; Garcia-Velasco et al., 2016; Hayashi et al., 2013; Kampe et al., 2018; Lahive et al., 2017; Luo et al., 2016; Shoults-Wilson et al., 2010; Tourinho et al., 2016; Velicogna et al., 2017), cependant des lacunes persistent sur les mécanismes de stockage de l'Ag, les sites et formes de stockage à l'intérieur de l'organisme. Chez les vers de terre, des hypothèses pouvaient être formulées du fait des connaissances actuelles sur la gestion des métaux internes et les rares informations trouvées dans la bibliographie. En effet, chez les vers de terre, les métaux tels que le Zinc, le Cuivre, le Cadmium ou encore le Mercure sont en partie régulés grâce aux métallothionéines (MTs), des protéines qui séquestrent ces métaux en les fixant via une liaison thiolée, afin d'empêcher les métaux d'exercer leur toxicité ou en vue de conduire ces métaux vers une expulsion par des voies naturelles (Demuynck et al., 2006; Vijver et al., 2004). Justement, deux études ont montré que lors d'exposition à l'Ag, des modifications de l'expression du gène codant pour la Cadmium Metallothionéine (gène *cdmt*) (Curieses Silvana et al., 2017; Hayashi et al., 2013) étaient observées, laissant penser que l'argent également était géré par séquestration par les MTs. De même, un papier évoque la localisation de l'Ag dans les vers de terre au sein des parois de l'intestin, des chloragocytes et des néphridies (Diez-Ortiz et al., 2015a), qui sont des zones d'accumulation principales des MTs chez les vers de terre et des organes de détoxication.

Ainsi, afin de poursuivre les recherches sur cette voie, une expérimentation a été pensée de manière à mesurer, dans un contexte d'exposition à des doses croissantes (de 33 à 277 mg $kg^{-1}$

m.s.) dans un milieu simplifié (sol artificiel OCDE), les doses d'Ag bioaccumulées chez les vers de terre *E. fetida*, la spéciation de l'argent bioaccumulé ainsi que la localisation de cet argent dans leur organisme. En parallèle, les traits de vie classiques tels que la mortalité et la reproduction ont été surveillés. Cette exposition a duré 4 semaines. L'article suivant, intitulé « Accumulation, speciation and localization of silver nanoparticles in *Eisenia fetida* earthworms» nous décrit cette expérimentation. Cette étude a mis en évidence la bioaccumulation de l'Ag chez les vers de terre, de manière non-proportionnelle à la quantité d'Ag présent dans le milieu mettant en évidence l'efficacité d'un mécanisme de régulation de l'Ag par l'organisme. L'Ag bioaccumulé dans les organismes se trouvait principalement dans le tissu chloragogène, les cœlomocytes et les tissus des néphridies. En outre, l'Ag est intégralement thiolé. Ainsi, du fait de sa localisation et de sa spéciation, il semblerait que l'Ag soit géré, comme d'autres métaux, par les MTs.

# Accumulation, speciation and localization of silver nanoparticles in the earthworm *Eisenia fetida*

**Pauline Courtois**[1], Agnieszka Rorat[1], Sébastien Lemiere[1], Clément Levard[2], Perrine Chaurand[2], Anna Grobelak[3], Christine Lors[1] and Franck Vandenbulcke[1*]

[1] *Univ. Lille, IMT Lille Douai, Univ. Artois, Yncrea Hauts-de-France, ULR 4515, - LGCgE, Laboratoire de Génie Civil et géo-Environnement, F-59000 Lille, France*

[2] *Aix Marseille Univ, CNRS, IRD, INRAE, Coll France, CEREGE, Aix-en-Provence, France*

[3] *Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland*

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**Abstract**

The use of silver nanoparticles (AgNPs) in agriculture and many consumer products has led to significant release of Ag in the environment. Although Ag toxicity in terrestrial organisms has been studied extensively, very little is known about the accumulation capacity and coping mechanisms of organisms in Ag-contaminated soil. In this context, we exposed *Eisenia fetida* earthworms to artificial OECD soil spiked with a range of concentrations of Ag (AgNPs or $AgNO_3$). The main aims were to (1) identify the location and form of accumulation of Ag in the exposed earthworms and (2) better understand the physiological mechanisms involved in Ag detoxification. The results showed that similar doses of AgNPs or $AgNO_3$ did not have the same effect on *E. fetida* survival. The two forms of Ag added to soil exhibited substantial differences in speciation at the end of exposure, but the Ag speciation and content of Ag in earthworms were similar, suggesting that biotransformation of Ag occurred. Finally, 3D images of intact earthworms obtained by X-ray micro-computed tomography revealed that Ag accumulated preferentially in the chloragogen tissue, coelomocytes and nephridial epithelium. Thus, *E. fetida* bioaccumulates Ag, but a regulation mechanism limit its impact in a very efficient manner. The location of Ag in the organism, the competition between Ag and Cu, and the speciation of internal Ag suggest a link between Ag and the thiol-rich proteins that are widely present in these tissues, most probably metallothioneins, which are key proteins in the sequestration and detoxification of metals.

**Keywords**

Silver, nanomaterials, earthworm, accumulation, speciation, X-ray absorption spectroscopy, X-ray micro-computed tomography

## 1. Introduction

Due to advances in nanotechnology and the increasing use of nanomaterials, metallic silver nanoparticles (AgNPs) are an emerging contaminant in the terrestrial environment (McGillicuddy et al., 2017). The incorporation of AgNPs in consumer products is increasing due to their unique properties, particularly their antimicrobial effects (Vance et al., 2015). Most silver (Ag) release occurs in municipal wastewater, and wastewater treatment plants allow efficient sequestration of Ag in sewage sludge (Kaegi et al., 2011). However, the Ag species trapped in these biosolids are subsequently spread on agricultural soil when sludge is recycled as fertilizer (Usman et al., 2012). A number of studies have shown that metallic Ag is transformed mostly into silver sulfide (Kaegi et al., 2013; Ma et al., 2014) and silver bound to thiols in the sewage system. This transformation strongly affects the behavior of Ag in the environment (Levard et al., 2012; Pradas del Real et al., 2017).

Another potential environmental exposure scenario in terrestrial ecosystems is the use of Ag as a nanopesticide or nanofertilizer via the direct application of metallic Ag to agricultural soils. Ag has bactericidal, fungicidal, insecticidal and herbicidal properties, and AgNPs have high inhibitory activity against crop pathogens (Chhipa, 2019; Khan and Rizvi, 2017). Moreover, AgNPs positively impact root elongation and the general growth of cultivated plants (Chhipa, 2019) when applied at concentrations between 1 and 200 ppm, depending on the plant species. However, at these concentrations, AgNPs are toxic to a variety of organisms.

The toxicological effects of AgNPs are quite well documented and include numerous impacts on soil microflora, flora and soil invertebrates (Courtois et al., 2019). The potential transfer of Ag in plants has received more attention (Yan and Chen, 2019) than transfer of Ag in animals. Most studies in animals have focused on life traits and protein changes in exposed animals (Yu et al., 2013). Although accumulation of Ag could be an important vector of the transfer of this metal in the trophic chain, studies of the underlying mechanisms are scarce.

In soil ecotoxicology, earthworms are widely studied based on their key role in most continental ecosystems and importance in the soil macrofauna. Earthworms participate in the maintenance of soil structure and fertility. In addition to enriching the soil with organic matter available for plants, they aerate the soil and promote water penetration by forming galleries during their burrowing activity (Bernard et al., 2010; Carbonell et al., 2009). Earthworms are highly consumed by birds, snakes, insectivorous mammals and rodents, especially during tillage. Consequently, accumulated contaminants can quickly move to upper trophic levels.

Studying Ag accumulation/defense mechanisms requires comprehensive knowledge of Ag distribution and speciation. In the present study, we investigated the accumulation, localization, and speciation of Ag (presented as NM-300K AgNPs or $AgNO_3$) in earthworms using several X-ray techniques. The main objective was to better understand how earthworms cope with Ag soil contamination in the context of silver pesticide/fertilizer use. For this purpose, *Eisenia fetida* earthworms were exposed to artificial OECD soil contaminated with a range of AgNP concentrations for 4 weeks. The effect of ionic Ag ($AgNO_3$) was investigated as a positive control. Biomass and mortality were followed, and Ag accumulation, speciation and localization in earthworms were measured. Finally, the accumulation mechanisms are discussed.

## 2. Materials and methods

### *2.1 Test species*

Genetically identified *Eisenia fetida* earthworms (Homa et al., 2015) from the laboratory breeding facility (LGCgE, University of Lille) were fed cow manure *ad libitum*. Adult earthworms, clitellated or not, were randomly selected and introduced into the microcosms after being weighed individually. The earthworms weighed 296 mg on average (min: 104 mg, max: 751 mg, mean standard deviation: 95 mg).

*2.2. Soil*

Artificial soil was prepared for this experiment according to OECD guideline n° 207 (OECD, 1984) and contained 10% sphagnum peat moss, 20% kaolin clay and 70% quartz sand. The soil pH was adjusted with calcium carbonate to 6 ± 0.5. Five weeks before adding the contaminant, 16.5 kg of soil was moistened with 6 liters of demineralized water. When the earthworms were added to the soil, water represented 27% of the weight of the wet soil.

*2.3. Silver species*

The standard reference material Ag-NM300K from the European Commission Joint Research Centre (JRC) was used as the AgNP source and was fully characterized in a previous work (Klein et al., 2011). Commercial NM300K-NPs were kindly provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME. Each bottle contained 2 g of NM300K diluted in dispersant with a volume of 2 mL. These metallic nanoparticles (NPs) were spherical and not coated and were dispersed in polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan mono-laurate (dispersant) with a nominal silver content of 10.2% by weight. Ninety-nine percent of the particles had a nominal size below 20 nm. Transmission electron microscopy indicated a mean size of 17 ± 8 nm. Smaller NPs of approximately 5 nm were also present (Mendes et al., 2015).

$AgNO_3$ solution was also prepared for comparison with exposure to Ag in ionic form. Silver nitrate salt ($AgNO_3$) was dissolved in sterile distilled water. The AgNPs and $AgNO_3$ solution were diluted with ultrapure water to obtain a final Ag concentration of 2 mg $mL^{-1}$.

*2.4. Experimental scheme (earthworm exposure)*

Earthworms in microcosms (with OECD artificial soil) were exposed to a range of Ag forms and concentrations. Four types of soil mixtures corresponding to 2 controls and 2 exposed conditions were prepared: control (soil only), dispersant (soil spiked with dispersant solution), AgNPs (soil spiked with AgNP solution) and $AgNO_3$ (soil spiked with $AgNO_3$ solution) (see Sup. Inf 1). Four different Ag concentrations, C1, C2, C3 and C4, were used for the AgNP and $AgNO_3$ microcosms: 30 (± 20), 70 (± 10), 120 (± 15) and 280 (± 40) mg $kg^{-1}$ (dry matter), respectively (these concentrations were chosen based on mortality rates reported in Garcia-Velasco et al. (2016) and Gomes et al. (2015)). Four different volumes of NM300K dispersant were used in the dispersant microcosms, which served as controls. These volumes were named D1, D2, D3 and D4 and corresponded to the dispersant volumes added in the AgNP microcosms for C1, C2, C3 and C4, respectively. Thus, a total of 13 microcosm conditions were established in triplicate. Ten earthworms were introduced in each microcosm, with a total of 390 earthworms. The exposure lasted 4 weeks. No food was added to the initial soil or during exposure.

*2.5. Analysis*

*Life traits*. Survival and biomass were measured. Biomass was followed by comparing the masses of the groups of organisms before and after exposure, and the results were expressed as the percentage loss.

*Metal concentrations in soils and accumulation in earthworms.* Immediately before exposure (T0), unexposed earthworms from the breeding facility were sacrificed to measure the metal concentrations present in the organisms. After exposure, earthworms were collected from each microcosm and placed in 1% agar for 24 hours for depuration (i.e. to remove the gut

content). Then, the earthworms were sacrificed by freezing for at least 48 hours and freeze-dried. The organisms were reduced to powder using liquid nitrogen and mineralized by digestion in acid medium (using $HNO_3$, $H_2SO_4$ and $HCl_4$ in a ratio of 10:2:3) as described by Bernard et al. (2010).

Soil samples were collected at the beginning (T0) and at the end of exposure (Tf = 4 weeks). These samples were freeze-dried and ground with a mortar and a 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 solutions obtained (mineralized earthworms and soils) were analyzed by ICP-OES (inductively coupled plasma-optical emission spectrometry) (Varian 720-ES, USA). The following classically studied metals were quantified: arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn) and Ag.

*Localization of silver in exposed earthworms using X-ray 3D imaging.* Two earthworms were imaged in 3D using X-ray micro-computed tomography (micro-CT): one non-exposed sample and one sample exposed to AgNPs (AgNP-C3) collected after 4 weeks in the dispersant and AgNP microcosms, respectively.

*Sample preparation*. After 24 hours of depuration in 1% agar, the earthworms were first anesthetized on ice and fixed for 16 hours in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. They were then dehydrated by soaking in a graded series of ethanol solutions (from 30% vol to 100% vol.) and subjected to supercritical point drying (Leica EM CPD300°). In this drying process, ethanol is replaced with liquid $CO_2$, which avoids the creation of damaging surface tension forces associated with drying by bringing the liquid in the sample to the gas phase without crossing the liquid-gas phase boundary. The dried samples were finally placed in polyimide tubing (Kapton).

*3D image acquisition.* 3D imaging of the earthworms was performed with a microXCT-400 X-ray microscope (Zeiss). High-resolution scans were acquired at 40 kV and 250 µA. A total of 2501 projections were collected through 360° rotation with an exposure time of 20 s per projection. A 20x magnification optical objective was selected to achieved an isotropic voxel of 0.9 µm and a field-of-view (FOV) of 0.9x0.9x0.9 $mm^3$. The FOV was centered at the lower end of the earthworm (rings 9 and 10 for the control and exposed earthworms, respectively) and included the coelomic cavity and the nephridia epithelium. The position of the FOV was selected from pre-visualization scans of the entire earthworm with lower spatial resolution (Supporting information 2). Volume reconstruction was performed with XMReconstructed-Parallel Beam-9.0.6445 software using a filtered back projection algorithm.

*3D image analysis.* Avizo 8.0 software (Hillsboro, OR, USA) was used for the visualization, processing, and analysis of the reconstructed dataset. The procedure developed in Chaurand et al. (2018) to isolate metal-based NPs was followed. Briefly, images of exposed and non-exposed (control) samples were compared after histogram x-axis normalization (i.e. colormap normalization). The histogram represents the X-ray attenuation in each voxel (expressed as an arbitrary gray scale value, GSV) of the analyzed volume as a function of the number of voxels for each GSV (intensity). The histogram x-axis was normalized using air as an internal standard. After the normalization step, the brilliant voxels in the image of the exposed sample that were not identified in the image of the control sample were attributed to the presence of Ag by thresholding (Supporting information 3).

*Speciation of silver in soils and earthworms*. Silver speciation in soil and earthworms was determined by X-ray absorption near-edge structure (XANES) spectroscopy, which permits the determination of the local atomic environment (speciation) of targeted atoms present in complex media. Silver K-edge (25.51 keV) XANES 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 earthworm 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 AgNPs, $AgNO_3$, $Ag_2S$, AgCl, Ag-thiocarbamate (Ag-thio), and Ag-humic acid (Ag-HA) was run in transmission mode. Normalization data reduction and linear combination fitting (LCF) were performed according to standard methods using Athena software (Ravel and Newville, 2005). The residual factor of LCF was calculated according to the formula $R = \sum(exp - fit)^2/\sum(exp)^2$, 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 higher than 10% among Ag species.

*2.6. Statistical analysis*

For biomass, mortality and metal content in earthworms, the majority of the data did not follow a normal distribution, and the variances were not homogeneous (Shapiro, Liliefors and Bartlett tests). Thus, Sheirer-Ray-Hare non-parametric tests and post-hoc tests based on ranks were used. For data following a normal distribution with homogeneous variances, ANOVA tests and Tuckey post-hoc tests were used. Correlation matrices (based on the Kendall method) were constructed. Tests were performed using the R package (R Core Team, 2018).

## 3. Results

*Metal quantities in soil*. At the initial time point, the concentrations of As, Cr, Cu, Ni, Pb and Zn were 1.08 (standard deviation 0.34), 1.05 (s.d. 0.30), 5.31 (s.d. 1.22), 0.62 (s.d. 0.38), 10.13 (s.d. 2.11) and 5.16 (s.d. 0.66) mg $kg^{-1}$, respectively. The concentration of Cd was below the detection limit. The Ag concentrations in the microcosms are shown in Table 1. As expected, there was no significant difference in Ag doses between the AgNP and $AgNO_3$ microcosms at each concentration (C1, C2, C3 and C4). Only very low concentrations of Ag were detected in the control and dispersant microcosms. One control microcosm appeared to have been very slightly contaminated by accident, but its Ag concentration remained negligible compared with the exposure conditions.

*Table 1 Silver content in the microcosms at the initial time point (mean in mg kg−1 of dry matter). The results were obtained by ICP analysis. Standard deviations are in parentheses*

| Concentration | **Control microcosms** | **Dispersant** | **AgNPs** | **$AgNO_3$** |
|---|---|---|---|---|
| C1 (or D1) | 1.95 (1.23) | 0.69 (0.97) | 26.33 (10.63) | 39.53 (20.66) |
| C2 (or D2) | | 0.61 (1.06) | 70.50 (8.15) | 71.03 (8.71) |
| C3 (or D3) | | 0.18 (0.31) | 109.57 (8.05) | 124.30 (7.59) |
| C4 (or D4) | | 0.00 (0.00) | 262.93 (27.87) | 290.90 (10.81) |

The concentrations of other metals were very low compared with Ag, which suggests that these metals would not hinder the accumulation of Ag by earthworms.

*Life traits*. A survival rate of 100% was observed in all of the control, dispersant and AgNP microcosms (Fig. 1). In the $AgNO_3$ microcosms, dose-dependent mortality was observed, with 6.7% mortality at the lowest concentration and 100% mortality at the highest concentration. During the 4 weeks of exposure, the earthworms lost weight in all microcosms,

including the controls. Thus, the loss of weight cannot be linked to Ag contamination. Biomass data for earthworms in the $AgNO_3$-C4 microcosm are absent due to total mortality.

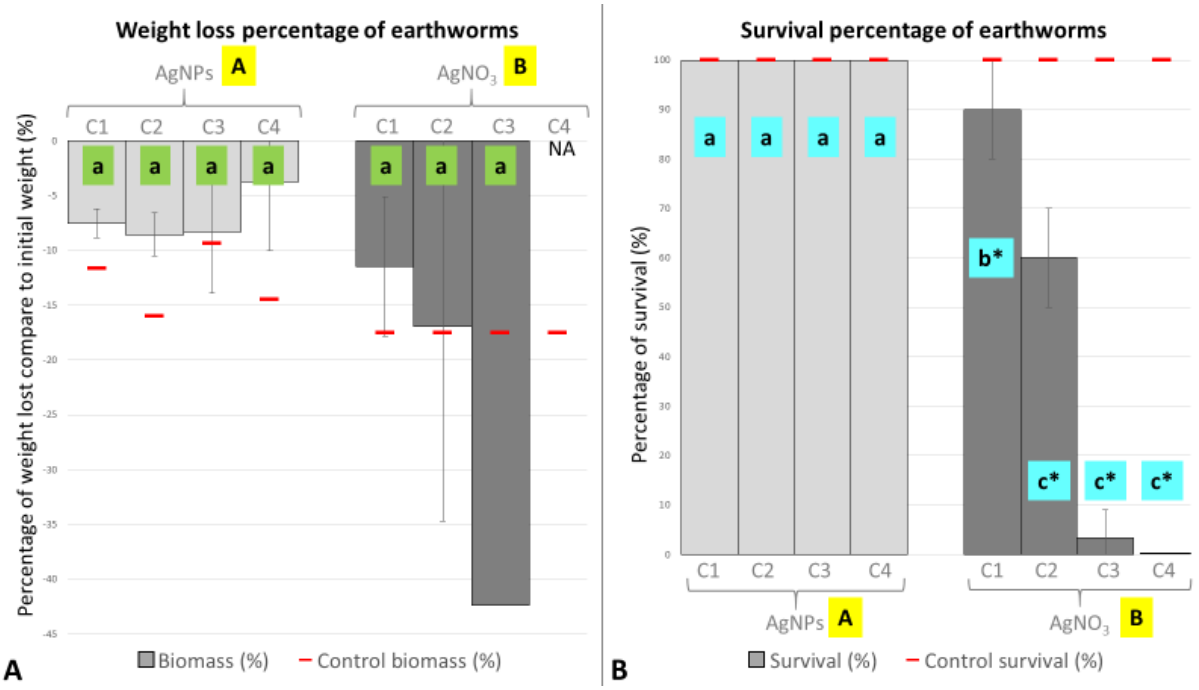


*Figure 1: A. Mean percentage of weight loss of the earthworm groups in the microcosms between the beginning and end of the experiment. B. Mean percentage of earthworm survival in the microcosms between the beginning and end of the experiment. The concentrations C1, C2, C3 and C4 correspond to mean concentrations of AgNO3 and AgNPs of 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg $kg^{-1}$ (dry matter) (the values were not significantly different between the two Ag sources). Asterisks (*) indicate statistically important differences between the Ag treatment and control. Lowercase letters in green indicate significant differences in biomass between the 4 doses of Ag (for one form of Ag). Lowercase letters in blue indicate significant differences in survival between the 4 doses of Ag (for one form of Ag). Uppercase letters in yellow indicate significant differences in biomass between the two forms of Ag (NPs or ionic), taking into account all concentrations. 'NA' indicates that biomass data were not available due to total mortality.*

*Metals bioaccumulation: silver*. At the end of exposure, the Ag content in earthworms varied among the different treatments (Fig. 2). Silver was not detected in organisms in the

control microcosms. In the presence of Ag, earthworms accumulated between 2.8 and 9.9 mg $kg^{-1}$ (average 5 mg $kg^{-1}$ of dry matter). The accumulation of Ag in the earthworms exposed to Ag was independent of the form of Ag (NPs or ionic) or the Ag dose in the microcosm. Data for earthworms in the AgNO3-C3 and AgNO3-C4 microcosms are absent because the insufficient quantity of material available for analysis due to significant mortality.

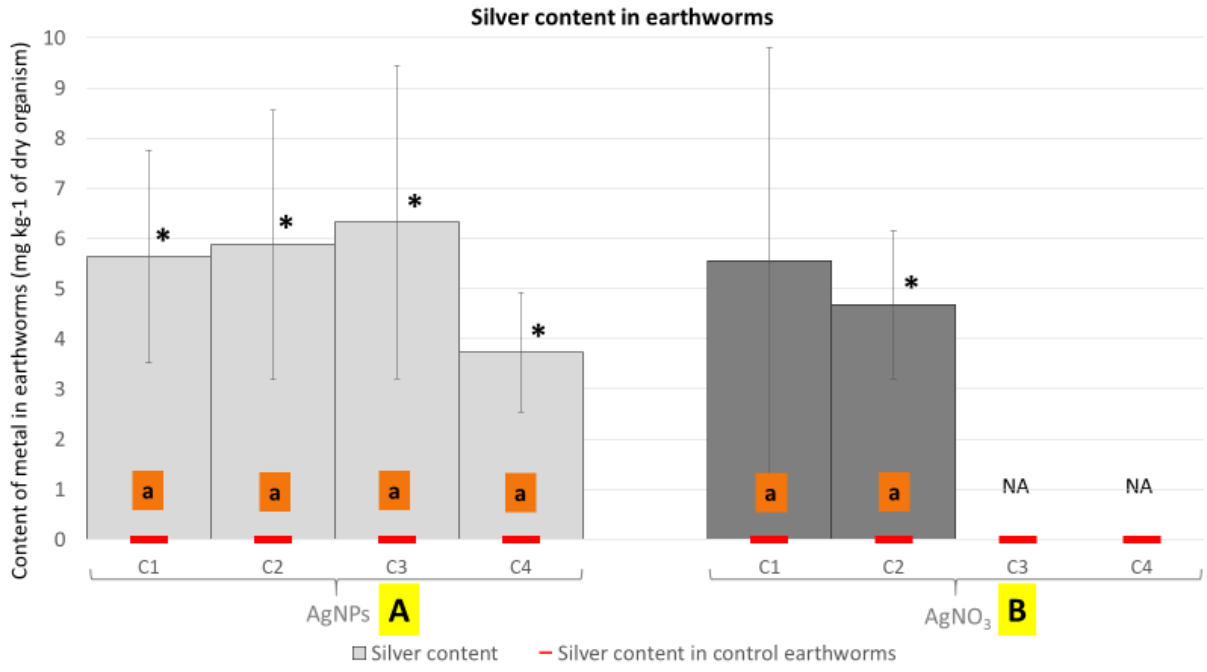


*Figure 2: Ag content in earthworm bodies (mg kg-1). "AgNPs" corresponds to microcosms with silver nanoparticles. "AgNO3" corresponds to microcosms with silver nitrate. The concentrations C1, C2, C3 and C4 correspond to 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg $kg^{-1}$ (dry matter) (the mean concentrations of AgNO3 and AgNPs were not significantly different). Stars (*) indicate significant differences from the associated control without silver. Lowercase letters in orange indicate significant differences in metal content between the 4 concentrations (C1, C2, C3 and C4) of Ag (AgNPs and AgNO3 were not compared). Uppercase letters in yellow indicate significant differences in Ag content between the 2 forms of Ag (NPs or ionic), taking into account all concentrations. 'NA' indicates that data were not available due to significant mortality.*

*Metals bioaccumulation: other metals*. No significant variations of metal quantities in earthworm bodies were observed for Cd, Cr, Ni, Pb and Zn compared with the corresponding control. A single significant difference in As concentration was observed between the $AgNO_3$-C2 microcosm and its control (Supporting information 2). The Cu concentration in earthworms was similar in all treatments without Ag (Fig. 3). In the $AgNO_3$-C2 microcosm (no results were obtained for the $AgNO_3$-C3 and $AgNO_3$-C4 microcosms because of earthworm mortality), there was a decrease in the Cu concentration, but this difference was not significant compared with the control. However, in the AgNP microcosms, earthworms accumulated significantly less Cu (approximately two times less) compared with the controls.

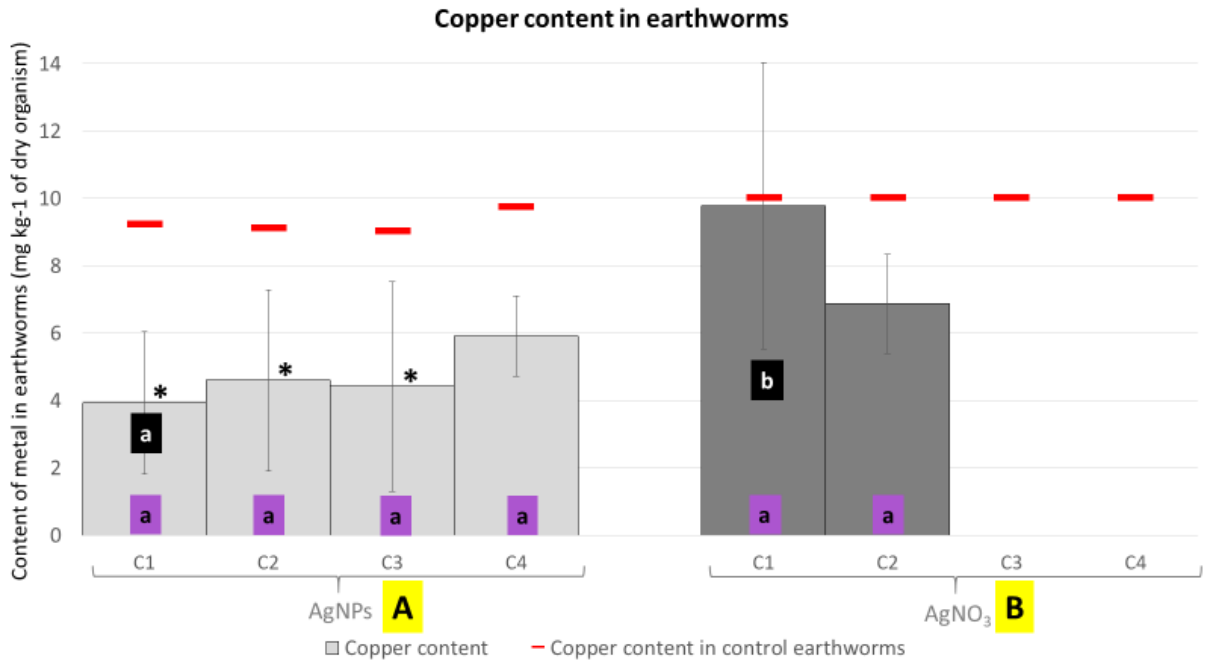


*Figure 3: Cu contents in earthworm bodies (mg kg-1). "AgNPs" corresponds to microcosms with silver nanoparticles. "AgNO3" corresponds to microcosms with silver nitrate. The concentrations C1, C2, C3 and C4 correspond to 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg $kg^{-1}$ (dry matter) (mean concentrations of AgNO3 and AgNPs, which were not significantly different). Stars (*) indicate significant differences from the associated control without Ag. Lowercase letters in purple indicate significant differences in metal contents among the 4 doses of Ag (for one form of Ag). Uppercase letters in yellow indicate significant differences in Cu content between the 2 forms of Ag (NPs or ionic), taking into account all concentrations. Lowercase letters in black indicate significant differences in Cu content between the 2 forms of Ag at one given concentration. 'NA' indicates that data were not available due to significant mortality.*

In summary, Ag was the only metal present in greater concentrations in earthworms exposed to AgNPs conditions than in those under control conditions.

*Localization of Ag in earthworms:* Brilliant voxels (i.e. voxels exhibiting high X-ray absorption) were observed in the micro-CT volume of exposed earthworms (AgNP-C3, with 109.57 (± 8.05) mg $kg^{-1}$ of AgNPs). Although this imaging technique cannot identify the source

of these brilliant voxels, they were not observed in the non-exposed earthworm volume (Dis-D3, in dispersant) and can therefore be attributed to Ag accumulation areas by thresholding (Fig. 4) due to the absence of differences in bioaccumulation for other elements with high densities (metals). Thresholding provides the distribution of these areas of brilliant voxels (colored in red) in the whole scanned volume. Ag accumulation areas/spots were observed around the digestive tract, in the coelomic cavity, in free cells in the coelomic cavity (coelomocytes) and in the nephridial epithelium (Fig. 4).

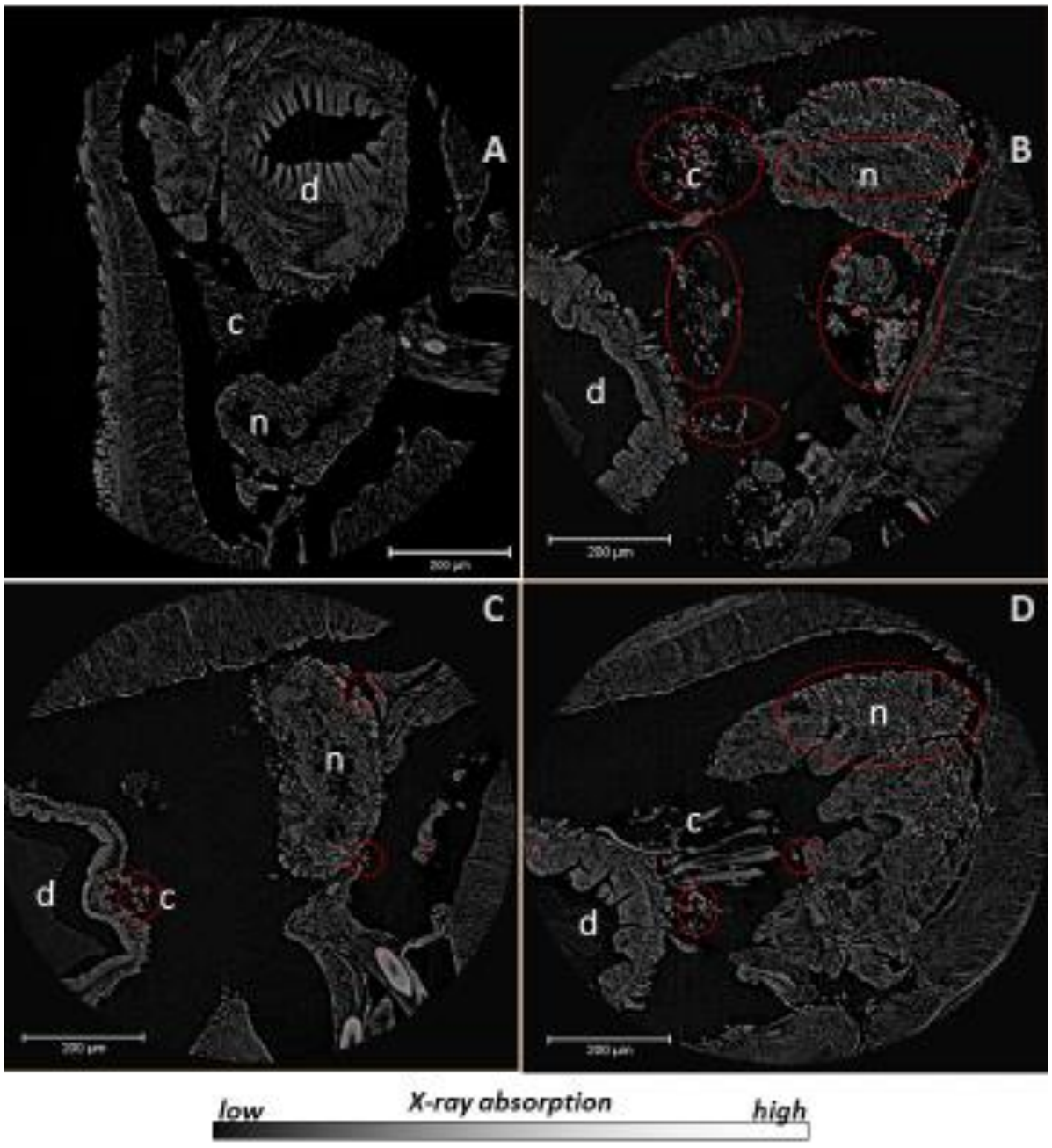


*Figure 4: Examples of 2D orthoslices extracted from the reconstructed volume of (A) non-exposed earthworms (Dis-D3) and (B, C, D) exposed earthworms (AgNP-C3). The digestive tract (d), free cells in the coelomic cavity (coelomocytes) (c) and the nephridial epithelium (n) are indicated. The pixels colored in red in (B, C, D) in dotted circles are brilliant voxels isolated by thresholding and associated with Ag. These brilliant pixels are not observed in (A). 1 px = 0.9 µm.*

*Speciation of silver*. Ag speciation in OECD soil after 4 weeks of incubation depended on the initial form (NPs or ionic). Ag initially spiked as AgNPs remained mainly metallic, but approximately 15% became complexed with natural organic thiols (Fig. 5). Ag initially spiked as $AgNO_3$ was linked with humic acid (52%) and organic thiols (33%), and approximately 15% was in metallic form. Regardless of the exposure scenario (AgNPs or $AgNO_3$), the speciation of Ag accumulated in earthworms was similar and consisted of Ag bound to thiols (Fig. 5).

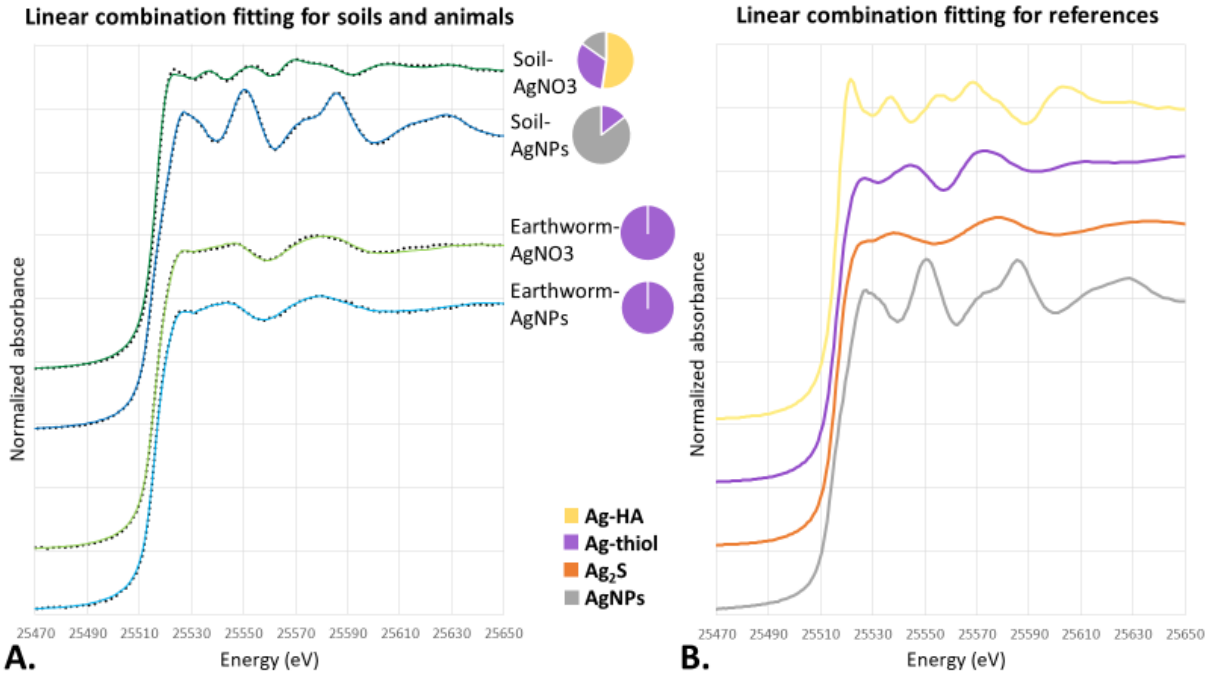


*Figure 5: A. Linear combination fitting (LCF) of the XANES spectra of the samples collected at different time points (dotted lines) and experimental spectra (solid lines) of soil and earthworms after 4 weeks of exposure to AgNPs and $AgNO_3$. The curves for the samples are colored as follows: dark green, soil spiked with $AgNO_3$; dark blue, soil spiked with AgNPs; light green, earthworms exposed to $AgNO_3$; light blue, earthworms exposed to AgNPs. B. XANES spectra of the model compounds used for LCF. Ag-HA (in yellow) was used as a proxy for Ag complexed to natural organic matter (humic acids). AgNPs (in grey) corresponds to the initial NM300K AgNPs used for the experiment. Ag-thiol (in purple) was used as a proxy for Ag bound to an organic thiol. $Ag_2S$ (in orange) corresponds to silver sulfide (acanthite mineral).*

## 4. Discussion

OECD soil is a simplified matrix for evaluating the effects of medium- and long-term exposure in a soil naturally deprived of many metals and other contaminants. In the present study, the use of OECD soil allowed the effects of added Ag and the underlying mechanisms to be explored under simplified experimental conditions. To prevent the possible ingestion of additional metals, no food was added to the medium. Use of this medium was therefore appropriate for the main objective of our work, which was to locate the sites of Ag accumulation by micro-CT.

High AgNP concentrations did not affect the life traits of *E. fetida* earthworms. The weight loss observed in the AgNPs microcosms was similar to that observed under control conditions and was due to a lack of food. OECD soil is poor in organic matter and nutrients, and food was not provided during the experiment. By contrast, dose-dependent toxicity of $AgNO_3$ resulting in weight loss and mortality was observed. Thus, the toxicity of $Ag^+$ was stronger than that of AgNPs, consistent with previous observations of *E. fetida* in both artificial (Diez-Ortiz et al., 2015a; Gomes et al., 2015; Heckmann et al., 2011) and natural soils (Novo et al., 2015). Higher toxicity of ionic Ag compared with AgNPs has been reported for many plants and animal species (Courtois et al., 2019), and there is a consensus that the toxicity of Ag is mainly due to its ionic form. We recognize that the starvation of the earthworms may have interfered with the results presented here. In the presence of optimal food, the effects of the two forms of Ag might be exacerbated or reduced.

Despite the differences in toxicity observed between the treatments, in all conditions with Ag (ionic or NPs), the mean bioaccumulation by earthworms in the body was 4 to 5 mg $kg^{-1}$ (dry matter). Thus, the form of Ag (NPs or ionic) had no influence on the amount of Ag bioaccumulation. In earthworms, metal accumulation is related not only to food intake but also to dermal absorption of dissolved ions (Vijver et al., 2003). Because of the differences in Ag

speciation in soil, one might expect Ag linked to organic matter (humic acids) to be metabolized more readily than thiolated Ag. When combined with the dermal absorption of $Ag^+$ ions, this increased metabolism could explain the higher toxicity of $AgNO_3$. However, (Diez-Ortiz et al., 2015b; Garcia-Velasco et al., 2016) showed that Ag is mainly internalized by soil ingestion. Our bioaccumulation results show that Ag can enter the earthworm body. Ag may also pass though the epidermis as dissolved $Ag^+$ ions. Unrine et al. (2008) demonstrated that dermal absorption of Au (gold) nanoparticles occurs in earthworms. However, as mentioned previously, dermal absorption is not the main route of metal internalization.

Contradictory results were reported by Shoults-Wilson et al. (2010) and Bourdineaud et al. (2019), who showed that *E. fetida* in artificial soil accumulated two to fifteen times more silver when exposed to $AgNO_3$ compared with AgNPs. Interestingly, these authors used AgNPs that were two to three times larger (between 50 and 60 nm) than the NM-300K AgNPs used in this study, which may have hindered the dermal absorption and metabolism of AgNPs. Supporting this hypothesis, Unrine et al. (2008) showed that the internalization of metallic NPs (Zn and Au) in *E. fetida* decreased with increasing NP size.

The Ag concentration in the soil did not influence Ag bioaccumulation by earthworms, which suggests that a very efficient regulation mechanism limits the internal content of Ag even when the environmental concentration is very high. A similar phenomenon (plateau and regulation) was reported by Coutris et al. (2011), who observed rapid Ag excretion from *E. fetida* after the end of exposure.

Regardless of the original form of Ag in the microcosms, the bioaccumulated Ag in earthworms was always bound to organic thiols. However, in OECD soil, even after 4 weeks of incubation, Ag speciation differed greatly between the AgNP and $AgNO_3$ microcosms, indicating biotransformation of Ag by earthworms. Since a regulation/excretion mechanism limits the Ag content in the body, it is likely that earthworms release Ag after biotransformation.

Thus, soil organisms like earthworms might change the speciation of Ag in the environment and, consequently, its availability.

3D images of entire earthworms obtained by micro-CT showed that Ag (originating from AgNPs) was stored and/or transiting in chloragogenous tissue, coelomocytes and the nephridial epithelium. A similar result was obtained by Diez-Ortiz et al. (2015b) by X-ray chemical analysis (micro-XRF) of the internal distribution in transverse sections: Ag was observed in the gut wall, liver-like chloragogenous tissue and nephridia. These cells and organs are related to immunity and detoxification functions. Chloragogenous tissue covers the outer part of the intestine and is considered to have a liver-like function. For instance, the chloragogenous tissue accumulates wastes produced by digestion and can sequester metals (Lapied et al., 2010; Morgan and Morgan, 1993; Vijver et al., 2004). Moreover, chloragogenous tissue plays a role in earthworm immunity (Fischer, 1993). Coelomocytes are immune cells involved in the elimination of foreign bodies by phagocytosis and encapsulation (Garcia-Velasco et al., 2017), and at least some coelomocytes are derived from chloragocytes (Hamed et al., 2002). Nephridia are organs involved in osmoregulation and excretion (Davidson et al., 2013).

In the present study, accumulation of Ag was concomitant with a decrease in Cu accumulation. Cu is an essential metal that is specifically stored by metallothioneins (MTs). MTs, stress proteins that bind essential and non-essential metals through thiolated bonds, participate in the homeostasis of essential metals such as Zn and Cu as well as non-essential metals such as Cd or mercury (Hg) (Demuynck et al., 2006; Vijver et al., 2004). Interestingly, in earthworms, MTs are preferentially but not exclusively localized in the epithelial cells of the intestine, chloragogenous tissue, coelomocytes and nephridia (Morgan et al., 2004). According to the localization of Ag observed by micro-CT, its internal speciation (linked to a thiolated molecule), and competition with Cu, Ag is probably bound by MTs in earthworms. The same

hypothesis was proposed by Baccaro et al. (2018), who also observed that bioaccumulated Ag was related to sulfur. Furthermore, in mice, Ag can bind to MT with higher affinity than Cu (Sugawara and Sugawara, 1984). Consequently, Ag probably displaces Cu from MT. Moreover, Hayashi et al. (2013) and Curieses Silvana et al. (2017) observed changes in the expression of genes encoding MTs in *E. fetida* exposed to AgNPs and $AgNO_3$ (in natural and OECD soil, respectively). Therefore, it seems that MTs have a role in the detoxification mechanisms of Ag. Taken together, these results suggest a pathway for the absorption, detoxification and excretion of Ag.

In summary, Ag is probably mainly taken up by ingestion, absorbed by the gut and at least temporarily stored in chloragogenous tissue before detoxification. For excretion, Ag must be transferred from chloragocytes to the nephridia. Two mechanisms can be proposed. First, MT-metal complexes are discharged from the coelomic cavity and then excreted by the nephridia. This mechanism is supported by the work of Nordberg (1989) and Morgan et al. (2004), who described the capacity of MTs linked to metals to enter excretory organs in mammals and earthworms. Second, Ag could be transferred to coelomocytes in the coelomic cavity and stored, inducing the gene encoding MTII (Brulle et al., 2008). Transfer of Ag into coelomocytes can occur via two pathways: transformation of chloragocytes that have stored Ag into free coelomocyte cells or release of metal-bound MT into the coelomic cavity by chloragocytes and subsequent uptake by coelomocytes. In the event of excessive ingestion of Ag that can be not managed conventionally by storage in proteins and excretion, another mechanism might help limit the levels of Ag in the body. For example, Roubalová et al. (2018) showed that when earthworms are confronted by aggression, earthworms can expel coelomic fluid with coelomocytes via the dorsal pores. This mechanism would quickly remove from the body a large amount of metals trapped in the coelomocytes.

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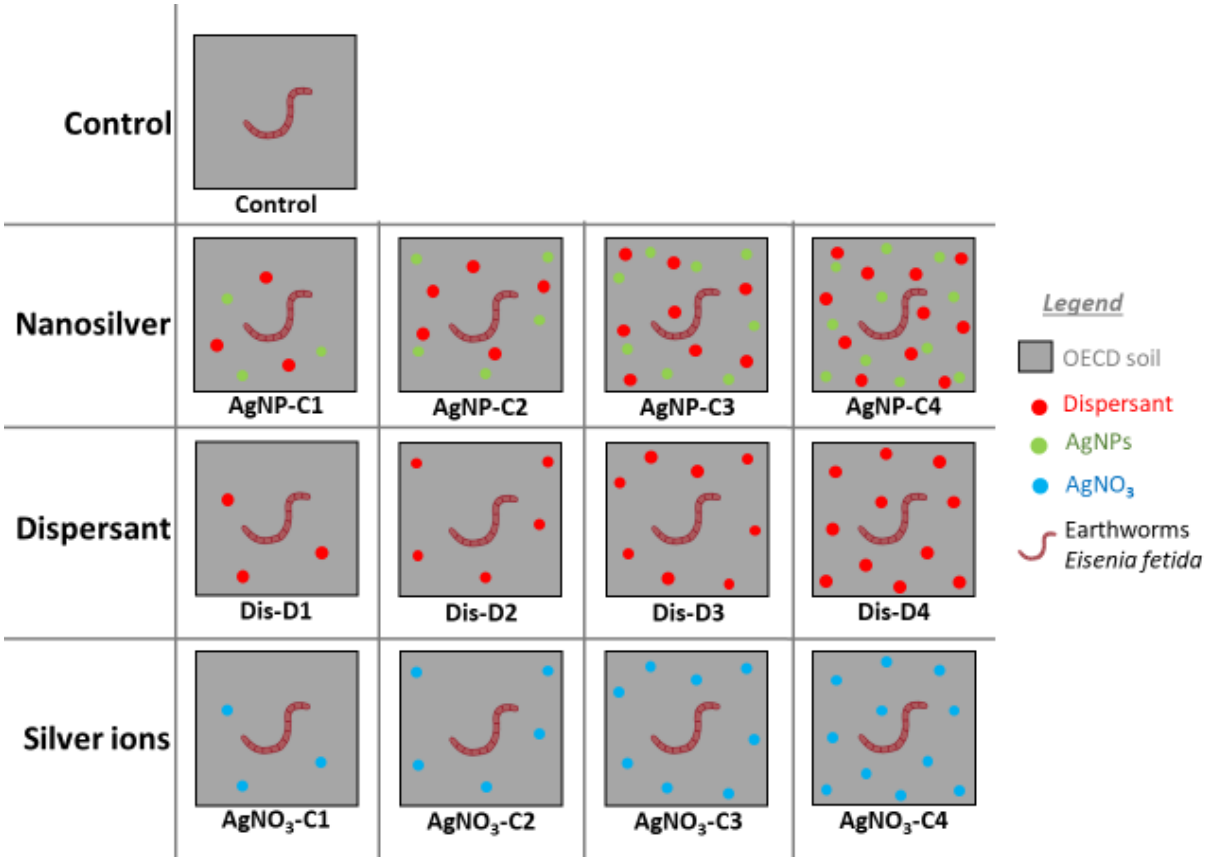
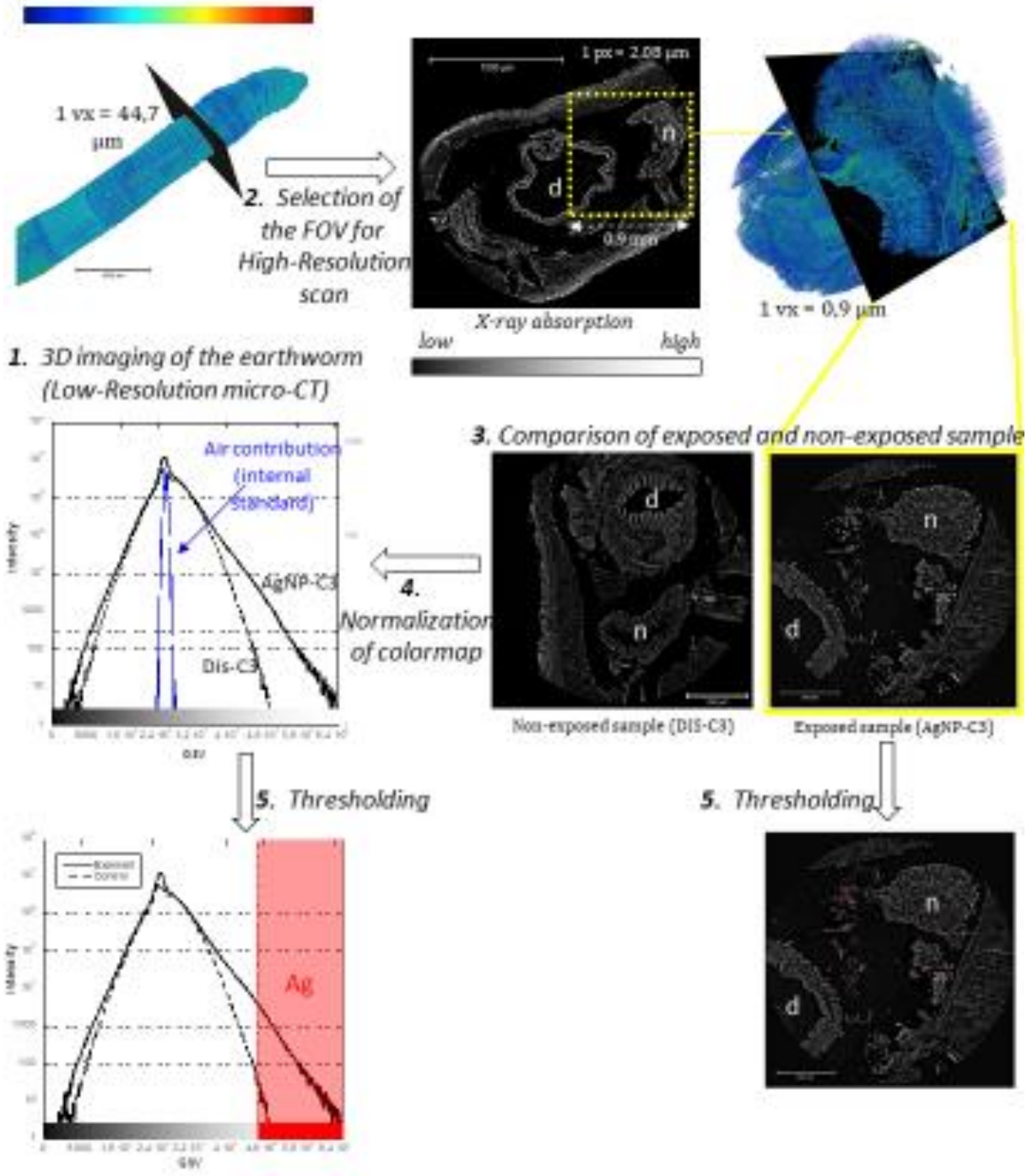
**Supplementary informations:**




*Sup. Inf. 1: Scheme of the experimental design. "Control" corresponds to the microcosm without any inputs. "AgNPs" corresponds to silver nanoparticles. "Dis" corresponds to dispersant. "$AgNO_3$" corresponds to silver nitrate. The concentrations C1, C2, C3 and C4 correspond to 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg $kg^{-1}$ of Ag (dry matter) (the mean concentrations of $AgNO_3$ and AgNPs were not significantly different). The volumes D1, D2, D3 and D4 correspond to the volumes of dispersant added to the microcosms. Dispersant was added in the same amount as in the corresponding AgNPs microcosms, that is, 1.599, 2.666, 5.331 and 10.662 mL.*

*Sup. Inf. 2: Metal contents in earthworms (mean in mg $kg^{-1}$). The results were obtained by ICP analysis. "Control" corresponds to the microcosm without silver addition. "AgNPs" corresponds to silver nanoparticles. "Dis" corresponds to dispersant. "$AgNO_3$" corresponds to silver nitrate. The concentrations C1, C2, C3 and C4 correspond to 33 (± 20), 71 (± 10), 117 (± 15) and 277 (± 45) mg $kg^{-1}$ (dry matter) (the mean concentrations of $AgNO_3$ and AgNPs were not significantly different). Stars (*) indicate significant differences between the condition with Ag and the associated control without Ag. Standard deviations are in parentheses.*

| Microcosm | As | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|
| $AgNO_3$-C1 | 33.94 (7.26) | 2.50 (0.51) | 0.11 (0.18) | 9.78 (2.46) | 0.16 (0.05) | 0.40 (0.45) | 102.25 (4.53) |
| $AgNO_3$-C2 | **20.99* (3.54)** | 1.48 (0.25) | 0.31 (0.54) | 6.87 (1.42) | 0.18 (0.20) | 0.73 (1.27) | 95.07 (5.16) |
| AgNP-C1 | 25.76 (4.76) | 1.47 (0.36) | 0.11 (0.18) | **3.94* (1.08)** | 0.07 (0.08) | 0.67 (1.16) | 101.36 (3.03) |
| AgNP-C2 | 26.87 (2.82) | 1.60 (0.06) | 0.00 (0.00) | **4.60* (0.72)** | 0.03 (0.03) | 0.02 (0.03) | 96.79 (3.80) |
| AgNP-C3 | 24.45 (1.86) | 1.78 (0.06) | 0.00 (0.00) | **4.42* (0.20)** | 0.02 (0.02) | 0.10 (0.17) | 91.92 (3.41) |
| AgNP-C4 | 24.40 (7.18) | 1.75 (0.63) | 0.00 (0.00) | 5.92 (1.65) | 0.06 (0.03) | 0.01 (0.02) | 93.38 (4.09) |
| Dis-D1 | 36.45 (4.66) | 2.08 (0.43) | 0.00 (0.00) | 9.22 (0.87) | 0.10 (0.03) | 0.03 (0.04) | 105.04 (0.98) |
| Dis-D2 | 36.29 (5.00) | 2.03 (0.25) | 0.00 (0.00) | 9.11 (1.98) | 0.12 (0.07) | 0.04 (0.07) | 103.47 (4.94) |
| Dis-D3 | 33.54 (4.70) | 1.95 (0.53) | 0.00 (0.00) | 9.03 (1.72) | 0.15 (0.12) | 1.02 (1.77) | 95.97 (2.55) |
| Dis-D4 | 36.35 (4.42) | 2.07 (0.48) | 0.00 (0.00) | 9.75 (0.44) | 0.08 (0.04) | 0.04 (0.05) | 101.09 (1.98) |
| Control | 42.73 (6.15) | 2.15 (0.35) | 0.00 (0.00) | 10.01 (1.20) | 0.13 (0.05) | 0.06 (0.03) | 104.80 (7.15) |




*Sup. Inf. 3: 3D imaging of an earthworm by micro-CT. (top) Selection of FOV for high-resolution micro-CT scan. (bottom) 3D image analysis procedure for isolating Ag accumulation areas (normalization and thresholding step).*

## References

At the end of the manuscript

# Chapitre III

# EFFETS ECOTOXICOLOGIQUES DE L'Ag CHEZ *Eisenia fetida* ET LES MICROORGANISMES DU SOL

Comme précisé dans la revue introductive précédemment, la communauté scientifique a besoin de davantage de données sur les effets de l'argent sur l'environnement et ses organismes reflétant des conditions réalistes. Pour cela, il est important de prendre en compte la spéciation de l'argent et sa concentration, la matrice d'expérimentation et la durée d'exposition des organismes. En effet, seuls quelques travaux ont étudié les invertébrés du sol en conditions réalistes, montrant une toxicité et accumulation moindre des $Ag_2S$ par rapport aux AgNPs ou ions $Ag^+$ chez le cloporte *Porcellio scaber* (Kampe et al., 2018) et les vers de terre *Eisenia fetida* et *Eisenia andrei* (Lahive et al., 2017; Velicogna et al., 2017). De même, les premières études sur l'effet des $Ag_2S$ sur les microorganismes du sol en condition réaliste montrent globalement un impact sur les communautés, avec certains groupes de microorganismes sensibles, ainsi qu'un impact sur leurs activités (Doolette et al., 2016; Durenkamp et al., 2016; Kraas et al., 2017; Schlich et al., 2018, 2017), mais ces données restent rares.

Nous avons alors pensé à réaliser une expérimentation qui permettait d'évaluer la toxicité de l'argent sous la forme dans laquelle il est réellement rejeté dans l'environnement, en concentration réaliste par rapport aux données de la bibliographie. Pour cela, nous avons prélevé de la boue dans une station d'épuration polonaise et nous l'avons placé en bioréacteur juste après y avoir ajouté une quantité raisonnable d'argent afin de réaliser une digestion anaérobie comme parfois réalisé en station d'épuration afin d'assainir les boues avant leur recyclage. L'argent transformé a ainsi été apporté dans un sol agricole naturel via sa matrice

habituelle, la boue d'épuration, en 3 différentes concentrations, afin d'évaluer sa toxicité sur les vers de terre *E. fetida* et les microorganismes du sol. L'exposition a duré 5 semaines afin d'avoir une vision à moyen terme sur les effets provoqués. L'article qui suit présente le design expérimental, les résultats ainsi que la discussion qui en découle.

Cette étude a permis de confirmer la transformation des nanoparticules d'argent en sulfure d'argent au cours de la digestion des boues d'épuration et la stabilité, au moins à moyen terme, de ce dérivé dans un sol naturel. Il a été montré que l'Ag sulfuré n'avait pas d'effet important sur les traits de vie des vers de terre *E. fetida* à ces niveaux de concentrations (12, 17 et 25 mg $kg^{-1}$), bien qu'une faible bioaccumulation fût visible. Les microorganismes du sol ont eux aussi été peu impactés par l'$Ag_2S$ puisque la respiration globale du sol n'a pas été modifiée bien que de légères modifications de communautés microbiennes aient été observées.

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

**Pauline Courtois**[1*], Agnieszka Rorat[1], Sébastien Lemiere[1], Rémy Guyoneaud[2], Eléonore Attard[2], Manon Longepierre[2], François Rigal[3], Clément Levard[4], Perrine Chaurand[4], Anna Grosser[5], Anna Grobelak[5], Malgorzata Kacprzak[5], Christine Lors[1], Agnès Richaume[6] and Franck Vandenbulcke[1]

[1] *Univ. Lille, IMT Lille Douai, Univ. Artois, Yncrea Hauts-de-France, ULR4515 - LGCgE, Laboratoire de Génie Civil et géo-Environnement, F-59000 Lille, France*

[2] *Université de Pau et des Pays de l'Adour, E2S UPPA, CNRS, UMR IPREM 5254, Environmental Microbiology, 64000, Pau, France*

[3] *Azorean Biodiversity Group, Centre for Ecology, Evolution and Environmental Changes (CE3C), Departamento de Ciências Agráriase Engenharia do Ambiente, Universidade dos Açores, PT-9700-042 Angra do Heroísmo, Açores, Portugal*

[4] *Aix Marseille Univ, CNRS, IRD, INRAE, Coll France, CEREGE, Aix-en-Provence, France*

[5] *Częstochowa University of Technology, Faculty of Infrastructure and Environment, Czestochowa, Poland*

[6] *LEM - Laboratoire d'Ecologie Microbienne - UMR 5557 - 69622 Villeurbanne, France*

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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.

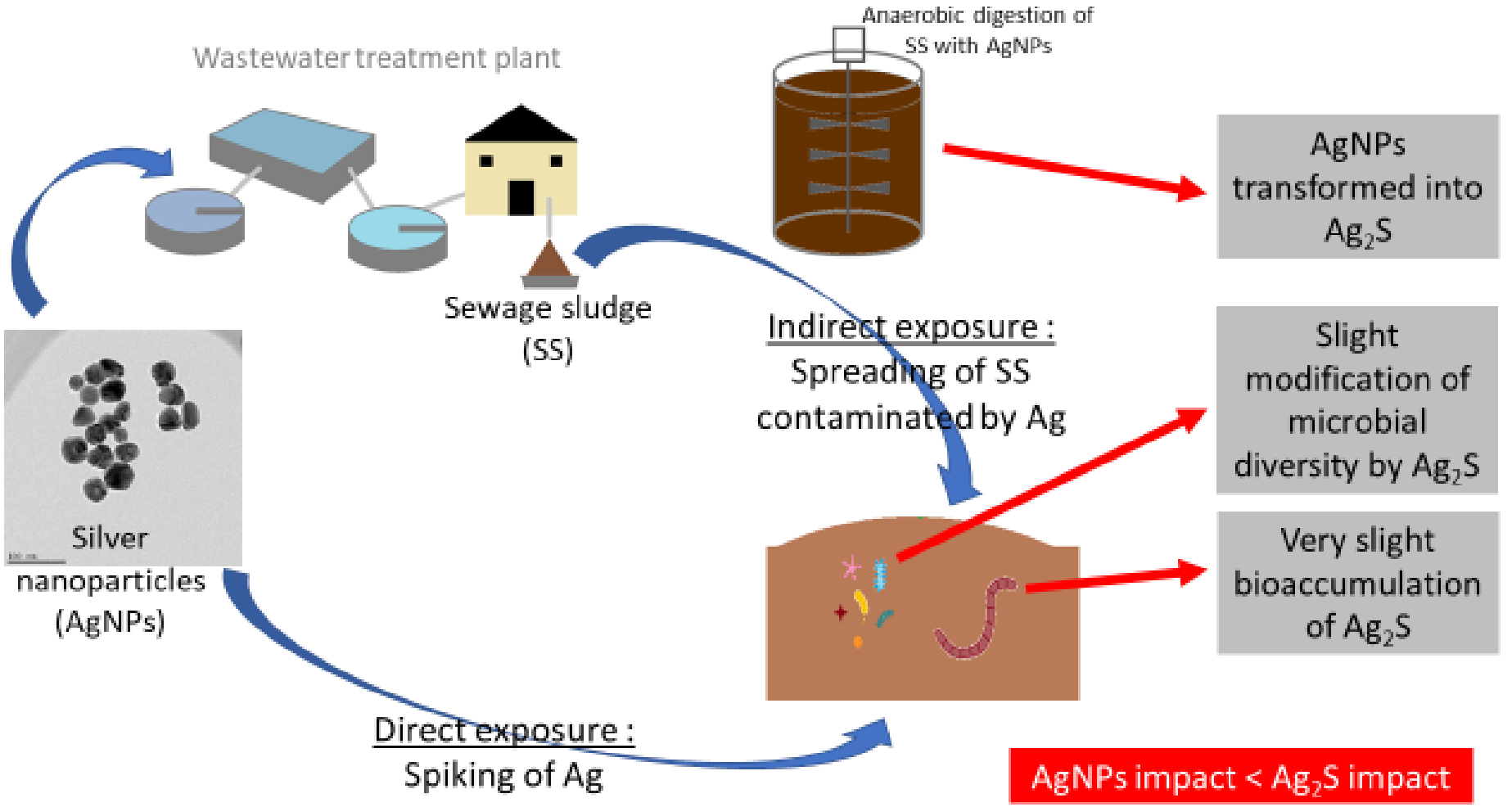
**Keywords**

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

**Highlights**

- Ag is brought to the agricultural soil from spreading of contaminated sewage sludge
- *Eisenia fetida* can bioaccumulate few amount of $Ag_2S$ brought by sewage sludge
- Ag in sludge has a slight impact on the diversity of soil microbial communities
- Speciation of Ag in sewage sludge causes less effect than nanoparticulate metallic Ag

**Graphical abstract**



## 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 in 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 understood 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., 2015a; Novo et al., 2015;

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

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

The main aim of this study was to assess the ecotoxicity of Ag introduced into the environment through SS land spreading. To get closer to a more realistic scenario, we performed controlled lab-scale anaerobic digestion of Ag-spiked SS collected from a WWTP that was subsequently spread at environmentally relevant doses on fresh agricultural soil. *Eisenia fetida* earthworms and soil microorganisms were then subsequently exposed for 5 weeks to: 1) soil spiked with AgNPs (with or without SS); 2) soil spiked with $AgNO_3$ (with or without SS); 3) control soil spiked with the dispersant used to disperse AgNPs (with or without SS); and 4) control soil without any additive (with or without SS).

The novelty of our study is the combined analysis of the responses 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) leached brown soil developed on wind-blown silts on chalky substrate, shallow to deep (DRAAF, 2013), classified as Luvisol (LV) according to WRB (World Reference Base) (Food and Agriculture Organization of the United Nations, 2015), 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 pH was and approximately 6.76 ± 0.01 (measure in KCl 1M according to ISO 10390:2005) and the total carbon and the dissolved organic carbon were 15.44 ± 1.34 mg $g^{-1}$ and 27.31 ± 1.34 mg $L^{-1}$. 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 mg $kg^{-1}$ 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 approximately 5 nm were also present (Mendes et al., 2015). The size distribution determinations by using zeta-sizer analysis resulted at about 100 nm (Klein et al., 2011). The commercial nanoparticle NM300K was kindly provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME (Schmallenberg, Germany). 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.

*2.5.2. Microcosm exposure - experimental mixtures*

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

a) Four of the treatments corresponded to indirect exposure, i.e., mixed with SS ("AD-X"). A realistic application quantity of SS was introduced to the microcosms in a single addition. In France, the maximum dose of SS spreadable over 10 years (Circular DE / GE n ° 357 of 03/16/99, 1999) was divided by 10, and quantities equivalent to 3, 6 and 10 times this calculated quantity were applied. The details of the selection of these quantities of SS for addition to the microcosms are given in SI 3. The three different dosages of SS were as follows: 60 g of fresh SS per kg of fresh soil as the 3-year perspective, "perspective 3" (3y); 120 g $kg^{-1}$ as the 6-year perspective, "perspective 6" (6y); and 200 g $kg^{-1}$ for the 10-year perspective, "perspective 10" (10y). The amount of Ag remaining in the SS after the fermentation process was estimated as 0.233 mg of Ag per g of fresh SS. Thus, the estimated amount of Ag was 14, 28 and 47 mg $kg^{-1}$ (fresh matter) for perspectives 3, 6, and 10, respectively (details of this estimation are provided in SI 4). The microcosms were filled with 1 kg of these mixtures.

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

- AD-dis: soil supplied with SS digested with dispersant solution consisting 4% w/w% each of polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan mono-laurate (AD-dis-3y, 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 after 1 day of incubation of the mixtures (corresponding to the time of initial depuration of the earthworms). The pH of the mixtures were 6.7 ± 0.1 in mixtures without SS and 6.5 ± 0.2 in mixtures with SS at the beginning of the experiment. 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. For that, the entire soil of each microcosm has been carefully sorted by hand. Survival was measured by counting earthworms that survived exposure. The biomass of the groups of earthworms was measured before (after emptying the intestines for 24 hours) 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 $\mu L^{-1}$ 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 sampled for the same purpose. Before that, a depuration phase of 24 h was conducted in order to empty the intestinal content. The organisms were frozen 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., 2010). 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 tests and post-hoc tests based on ranks were used. When data fulfilled statistical assumption of parametric tests (normality and homogeneity of variances), analysis of variance (ANOVA) and Tukey HSD (honestly significant difference) post-hoc tests were used. Correlation matrices (based on the Kendall method) were constructed.

For microorganism analyses, one-way ANOVA and Tukey tests were performed with the software PAST (Hammer et al., 2001) to determine if there were significant differences in respiration between the treatments. Microbial community composition was analyzed using PRIMER software (PRIMER-E Ltd., Plymouth, UK). Dissimilarity in OTU composition between all pairs of microbial communities was computed using Bray-Curtis distance and nonparametric permutational multivariate analysis of variance (PerMANOVA) (Anderson, 2001) was conducted to test for difference in composition among treatments. PerMANOVA was performed using permutation tests with 9,999 iterations. Results of the PerMANOVAs were visualized using non-metric multidimensional scaling ordinations (NMDS) based on Bray-Curtis distances. Finally, an indicator analysis (IndVal) (Dufrene and Legendre, 1997) was carried out to estimate the degree of association between OTUs and treatments. The analysis quantifies the fidelity and specificity of species (OTU) in relation to treatments or to groups of treatments, i.e., with or without SS on one hand and with or without Ag on the other

hand, and tests for the statistical significance of the associations using permutation tests with 9,999 iterations (De Cáceres et al., 2010; Dufrene and Legendre, 1997). The indicator value of species i for class j is obtained using the equation $IndVal_{ij} = 100 \cdot A_{ij} \cdot B_{ij}$, where $A_{ij}$ is specificity, i.e., the proportion of the individuals of the species i that are in the class j, and $B_{ij}$ is fidelity, i.e., the proportion of sites in the class j that contain the species i.

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

## 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%).

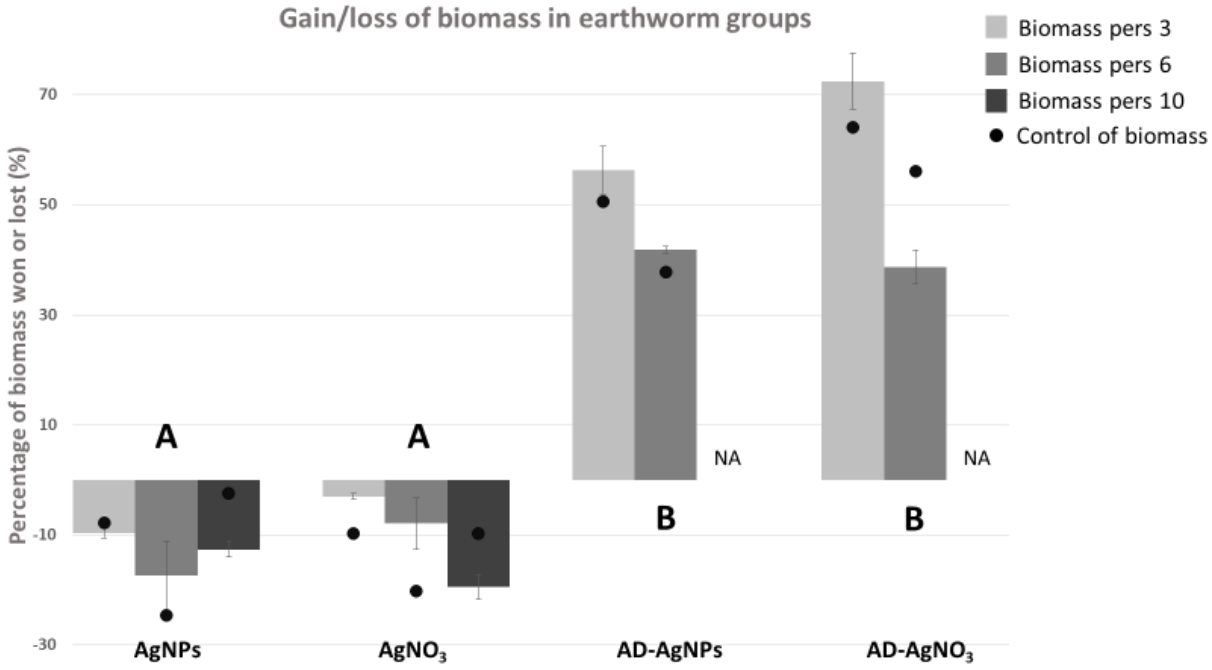



*Figure 1: Percentage of weight gain or loss in the groups of earthworms in the microcosms between the beginning and end of the experiment (percentages are reported to compensate for differences in initial weight). Each perspective is represented by a color: light grey for perspective 3, medium grey for perspective 6 and dark grey for perspective 10. The black dots correspond to the mean earthworm biomass under control conditions (dispersant for AgNPs, control for $AgNO_3$, AD-dis fir AD-AgNPs and AD-control for AD-AgNO3). NA corresponds to non-available data due to the high mortality in these conditions. The big letters show the differences between conditions AgNPs, $AgNO_3$, AD-AgNPs and AD-$AgNO_3$ (all perspectives combined). There were no differences between perspectives within the same condition.*

3.1.2. Gene expression levels in earthworms

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

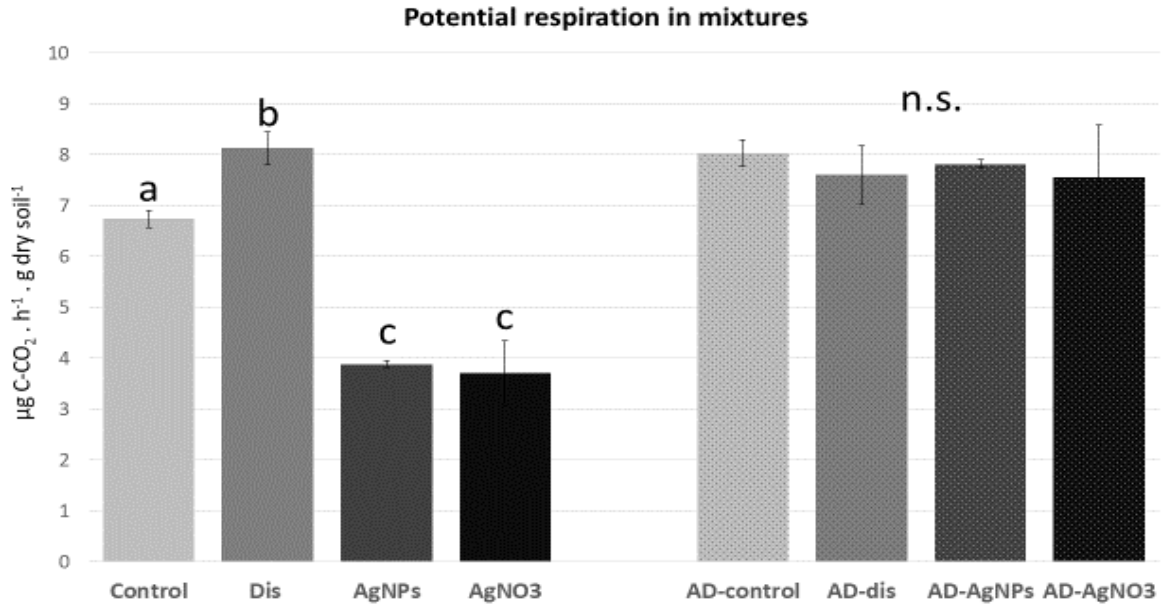



*Figure 2: Potential carbon respiration of microbial communities in mixtures (µg C-$CO_2$.$h^{-1}$.$g^{-1}$ of dry soil). The letters indicate significant differences in respiration between the 4 treatments without sewage sludge. "n.s." indicates that there were no significant differences between the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

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

### *3.2. Physicochemical analysis*

3.2.1. Metal content in mixtures

The soil concentrations of Ag were lower than expected (Table 3). There was no significant difference between these contents at the start and at the end of the experiment. The average soil Ag concentrations in perspectives 3, 6 and 10 were 11.56 ± 1.69 mg $kg^{-1}$, 16.97 ± 3.99 mg $kg^{-1}$ and 25.38 ± 3.69 mg $kg^{-1}$, respectively. In perspectives 3 and 10, the Ag concentrations in AD-AgNPs, AD-$AgNO_3$, AgNPs and $AgNO_3$ were similar. In perspective 6, there was a slight difference in Ag concentrations between the treatments with direct addition of Ag and those with addition of Ag via SS. For information, contents of other metals are precised in Table SI 9.

*Table 3: Silver concentration in mixtures at the end of the experiment (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 | | |

For information, the pH of the mixtures did not move a lot during the 5 weeks of experiment. The final pH of mixtures were 6.9 ± 0.0 in mixtures without SS and 6.2 ± 0.2 in mixtures with SS.

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.

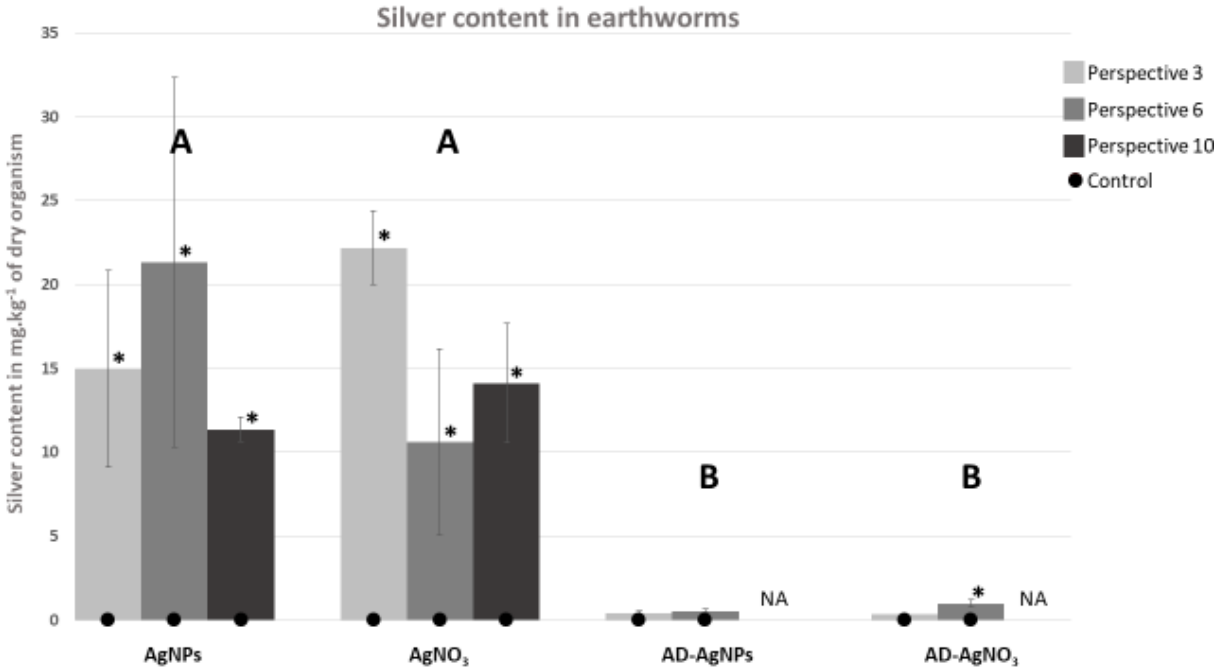



*Figure 4: Silver content in earthworms (mg of Ag $kg^{-1}$ of dry matter). Each perspective is represented by a color: light grey for perspective 3, medium grey for perspective 6 and dark grey for perspective 10. NA means data are non-available due to the high mortality in these conditions. The big 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.

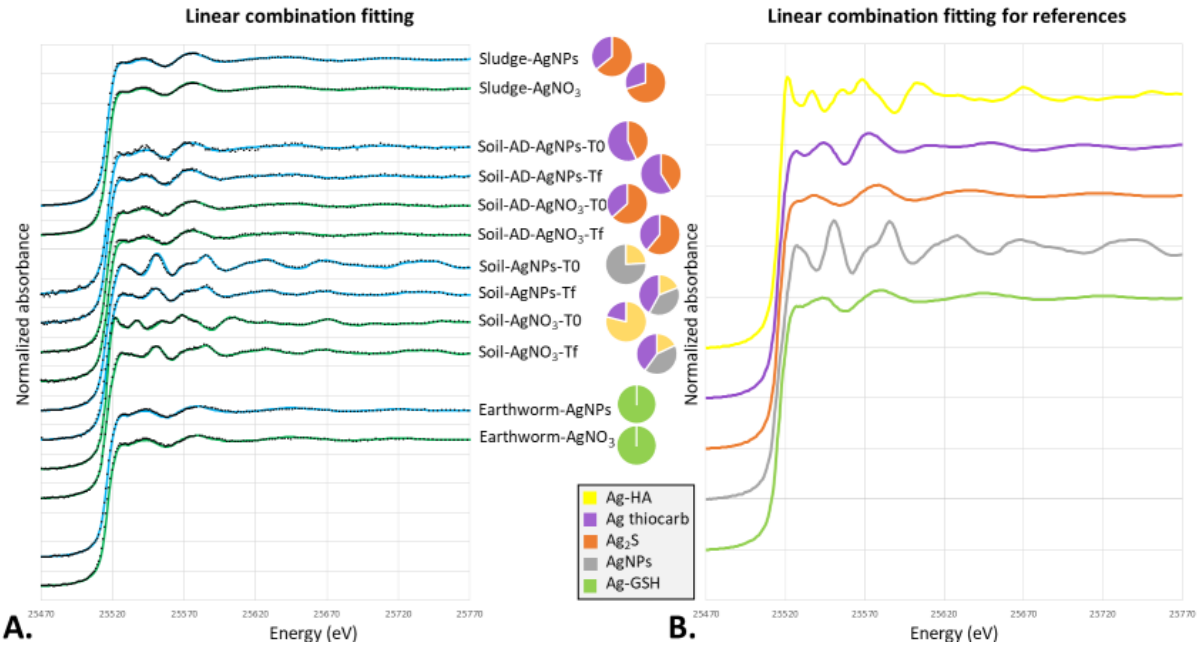



*Figure 5: A. Speciation of silver in sewage sludge, soils and mixtures at the beginning (T0) or end (Tf) of the soil incubations and speciation of silver in earthworms: linear combination fitting of XANES spectra is shown on the left (dotted lines correspond to experimental data, and solid colored lines 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

Investigation of Ag speciation in earthworms was possible only for the soil incubated without SS since the content of bioaccumulated Ag in earthworms exposed to the AD-AgNPs and AD-$AgNO_3$ microcosms was too low to be analyzed by X-ray absorption spectroscopy (Figure 4). The speciation of Ag bioaccumulated in earthworms was similar regardless of the initial form of Ag when exposed to AgNPs or $AgNO_3$. The XANES spectra were identical to those of the Ag-thiol model compound (Figure 5).

**4. Discussion**

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

earthworm survival. It is known that SS may contain high amounts of ammonium, which may be toxic for earthworms (Rorat, 2015).

The accumulation of Ag by earthworms did not depend on the Ag concentration in the soil or the initial form of Ag (NPs or ionic). Direct supply of Ag (AgNPs or $AgNO_3$) led to 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). Moreover, the difference of organic matter between microcosms with and without SS, may have played a role in the bioavailability of Ag. Indeed, Ag can complex with humic acids making Ag less bioavailable and therefore less toxic to the organisms (McKee et al., 2019). Likewise, the very slight difference in pH between the mixtures with and without sludge may have contributed to a slight difference in the bioavailability of the metal for the worms since a more acidic pH results in a higher bioavailability of Ag in the soil (Topuz and van Gestel, 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 first few days (between 1$^{st}$ and 7$^{th}$ day) of exposure with Ag. In artificial and natural soils, *cdmt* seems to be mainly affected by the high Ag contents (Choi and Park, 2015; Hayashi et al., 2013) but in Curieses Silvana et al. (2017) low (0.05 mg kg-1) and high concentrations caused this upregulation. In some cases, when the exposure lasts longer (10 – 14 days), downregulation of *cdmt* can be observed with low and medium concentrations (0.05 to 50 mg kg-$^{1}$) in artificial soil (Bourdineaud et al., 2019; Curieses Silvana et al., 2017). In all these cited studies, the measure of relative expression levels of these 3 genes were measured during the first 2 weeks of exposure. Short-term and long-term defense mechanisms may vary, and it is possible that by observing the expression of the genes after 5 weeks we missed earlier changes in the expression.

The results of this study showed that the Ag bioaccumulated in earthworms was bound to organic thiols, consistent with a previous study (Baccaro et al., 2018) and observations in *E. fetida* in a different context of exposure (Courtois et al., 2020a). The localization of Ag in organisms, its speciation, and Cu-Ag competition for bioaccumulation observed in the latter studies, as well as studies on the role of metallothionein (MT) (Demuynck et al., 2006; Morgan

et al., 2004; Sugawara and Sugawara, 1984; Vijver et al., 2004) and changes in the expression of MT-encoding genes in Ag exposure contexts (Curieses Silvana et al., 2017; Hayashi et al., 2013), suggest that Ag in earthworms are linked to MT. Regardless of the Ag concentration in soil (perspectives 3, 6, and 10), bioaccumulation in earthworms was similar, suggesting the existence of a regulation mechanism to limit the accumulation and therefore the toxicity of Ag. Such a regulation mechanism in earthworms might involve MT. Thus, by observing all published results, it would seem that the metalloproteins play an early role (first few days / weeks) in the detoxification of Ag (metallic or ionic) in *E. fetida*, hence an overexpression of the gene at the start of exposure. Then the expression of the gene returns to normal, probably because other defense mechanisms take over, which would explain that in the study below, at 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 or the high decomposition of the SS may have masked the respiration activity.

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 $AgNO_3$ ultimately had the same speciation and therefore similar effects on *E. fetida* and soil microorganisms. Finally, at low doses, Ag sulfides are less bioavailable than $Ag^+$ or AgNPs to certain organisms (Courtois et al., 2019), like earthworms (Lahive et al., 2017). The SS initially added with AgNPs and $AgNO_3$ essentially contained Ag sulfides at the end of fermentation. Anaerobic treatment of SS thus reduces the toxicity of Ag to organisms.

## 5. Conclusion

In conclusion, after 5 weeks, no strong effect of Ag on the microbial community and earthworms was observed when Ag was supplied to the soil via a reasonable quantity of SS. Speciation did not differ between AgNPs and $AgNO_3$ after the major chemical changes that occurred during fermentation. Interestingly, the microbial communities seemed to be highly resistant to Ag species supplied with sewage sludge, and the earthworms seemed to accumulate

much less Ag due to reduced bioavailability. The effects of Ag observed in the absence of SS were strongly limited when Ag was previously sulfided due to the digestion of SS.

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

The authors declare no competing financial interest.

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**Supplementary material**

*SI 1: Justification for choosing the silver concentration in the bioreactors*

According to a report from the EPA (U.S. Environmental Protection Agency) published in 2009 (U.S. EPA, 2009), the average content of Ag in SS varies between 2 and 856 mg $kg^{-1}$. In this study, we decided to use a final concentration of 40 mg $L^{-1}$ in bioreactors containing a final volume of 6 L. Yang et al. (2013) investigated the possible impact of high doses of Ag on the anaerobic digestion (AD) process (anaerobic glucose degradation, SS digestion, methanogenic assemblages). They observed that high doses of Ag (up to 40 mg $L^{-1}$) did not cause any significant differences in biogas and methane production, and the process was not affected. The concentration of the AgNP NM-300K stock solution was 2 mg $L^{-1}$. Thus, 120 mL of solution was added to the "AD-AgNPs" bioreactor in order to add 240 mg of Ag. A solution of $AgNO_3$ was prepared at the same concentration of Ag, and the same quantity of solution was added to the "AD-$AgNO_3$" bioreactor. For the "AD-dis" SS, 120 mL of dispersant was added. The AD-control condition did not contain any additive.

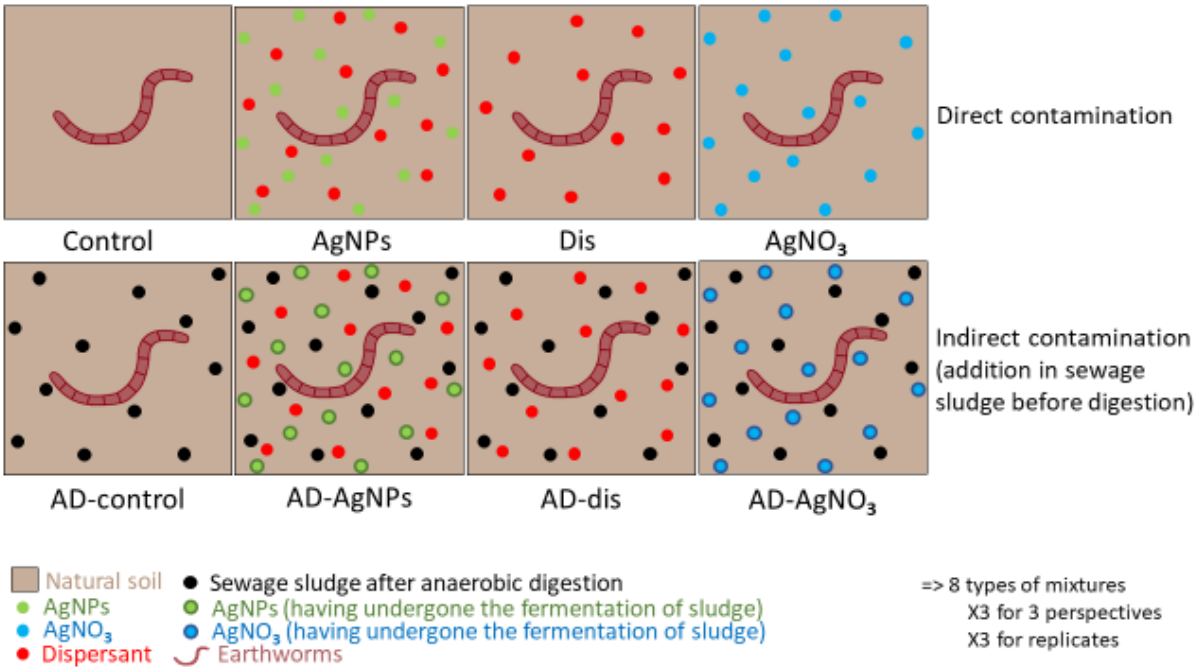


*Figure SI 2: Scheme of the experimental design*

*SI 3: Justification for choosing the sewage sludge doses in the microcosms*

The doses of SS introduced in the microcosms were chosen according to french regulations, more severe than the European legislation (Directive 86/278/ECC, 1986), which states that it is possible to apply up to 30 tons of dry matter per hectare for a period of 10 years (Circular DE / GE n ° 357 of 03/16/99, 1999). The density of soil used in this study was 1.25 g.cm$^3$, and the average depth of incorporation of SS into agricultural soil is approximately 8 cm (typically 5 to 15 cm). Thus, the soil mass that is mixed with SS over one hectare corresponds to approximately 1000 tons (density = mass/volume). Therefore, from a one-year perspective, it is possible to apply 3 tons of SS (dry matter) per 1000 tons of soil, i.e., 3 g of SS per kg of soil. Assuming that dewatered SS still contains approximately 85% water, the mass allowed for spreading is close to 20 g of fresh SS per kg of soil per year. However, European legislation allows the addition, at one time, of significantly higher SS quantities on agricultural soil, since it is possible to add up to 30 tons of SS per hectare per decade. It is therefore legally possible to apply the equivalent of several years at one time.

*SI 4: Justification for estimating the Ag concentration in sewage sludge.*

Literature showed that SS treatment traps approximately 90% of Ag in biosolids (Kaegi et al., 2011; Tiede et al., 2010). In our study, approximately 216 mg of Ag (of the 240 mg initially added) should have remained in the SS. After one month of fermentation, there was approximately 910 g of fresh SS in each bioreactor. Consequently, the addition of 60, 120 and 200 g of fresh SS per kg of fresh soil in the microcosms corresponded to the addition of 14, 28 and 47 mg $kg^{-1}$ of Ag depending on the time perspective.

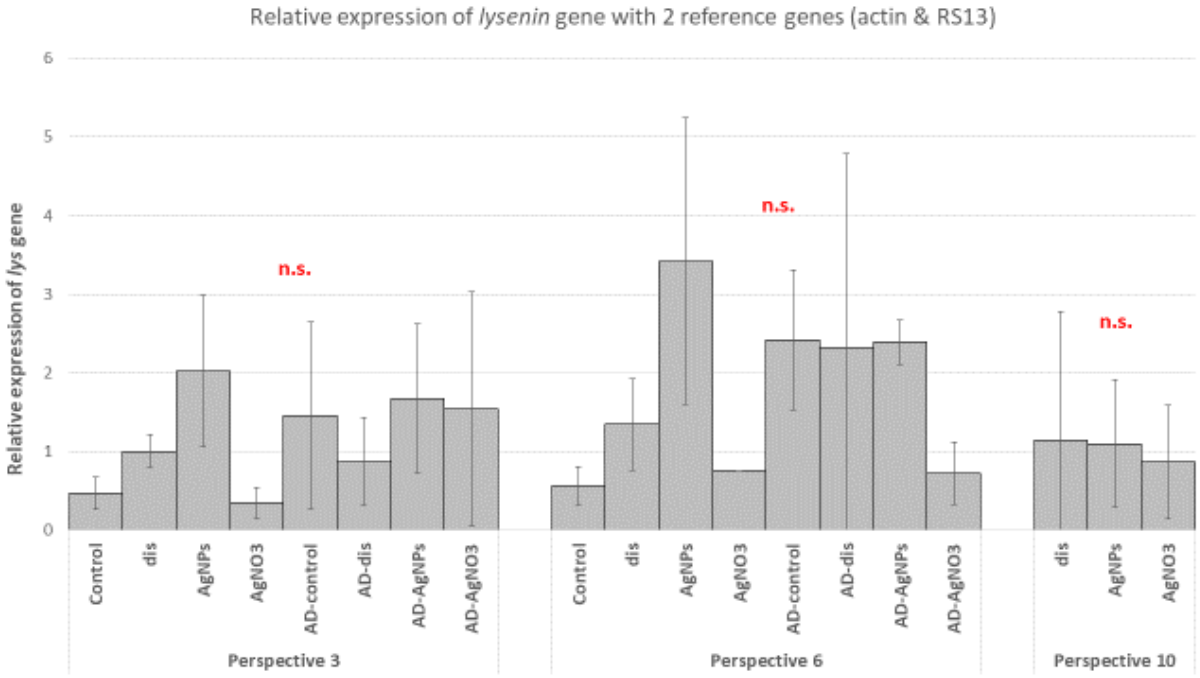


*Figure SI 5: Relative expression of the lysenin gene as determined by real-time PCR. "n.s." means no significant diffrences i.e. for each perspective, no significant differences were observed between all conditions.*

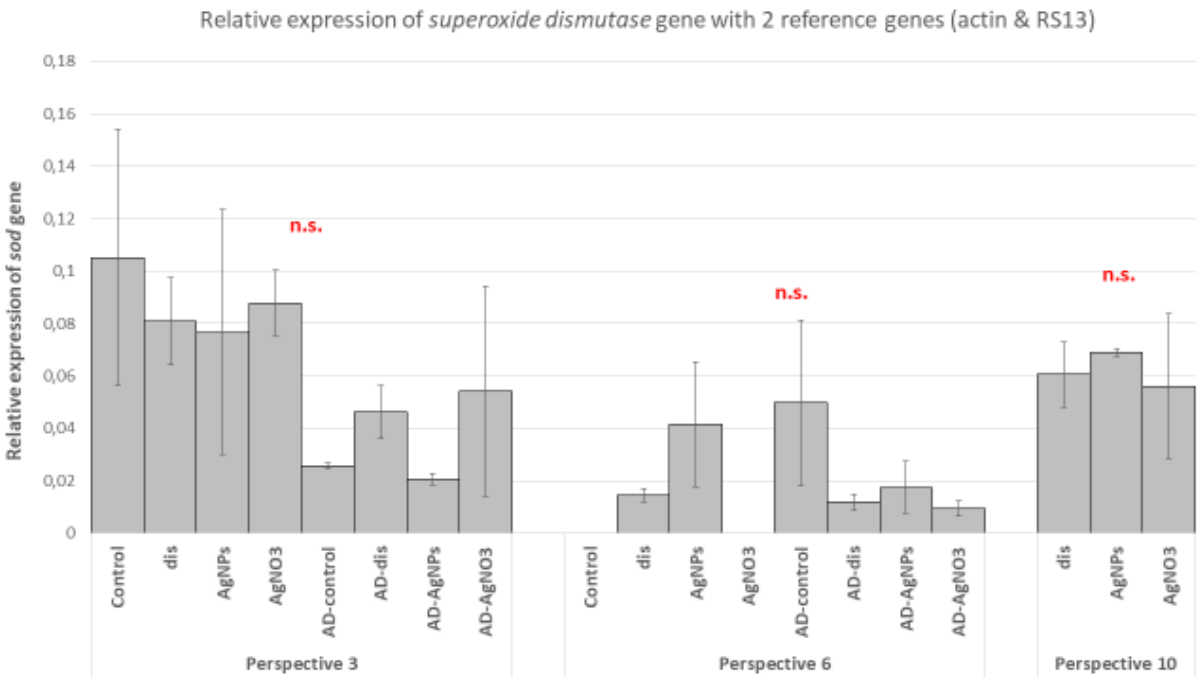


*Figure SI 6: Relative expression of the superoxide dismutase gene as assessed by real-time PCR. "n.s." means no significant diffrences i.e. for each perspective, no significant differences were observed between all conditions.*

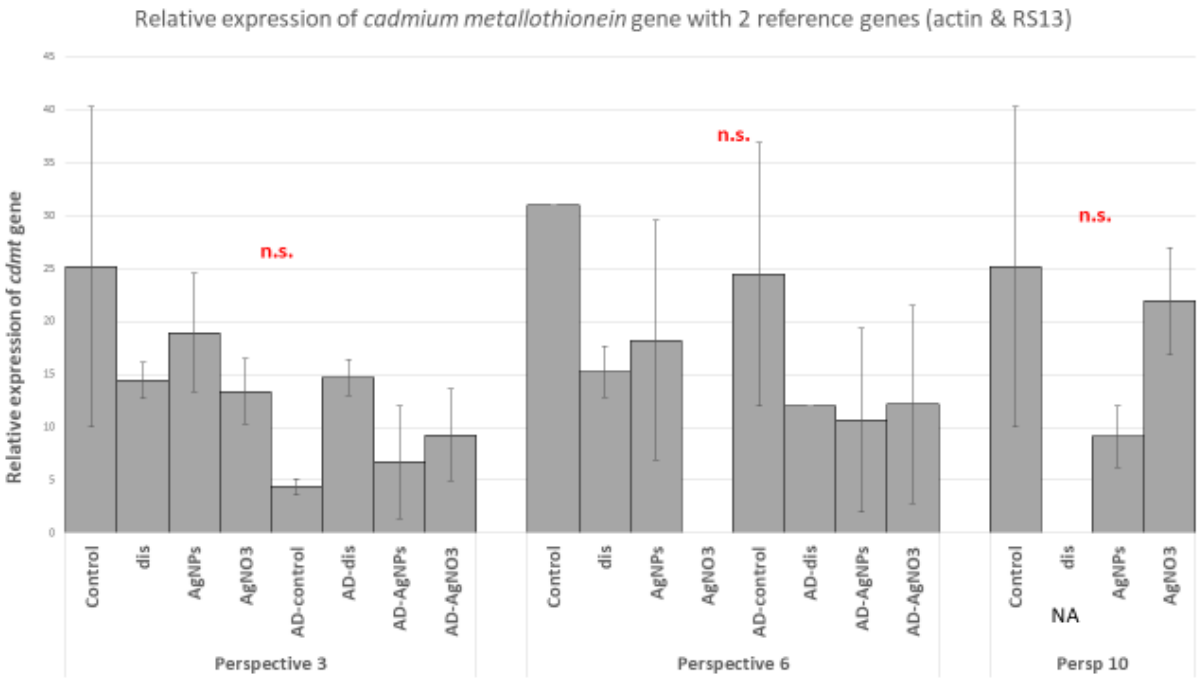


*Figure SI 7: Relative expression of the cadmium metallothionein gene as assessed by real-time PCR. NA means that data is not missing. "n.s." means no significant diffrences i.e. for each perspective, no significant differences were observed between all conditions.*

*Table SI 8: Results of IndVal analysis.*

| Taxa affiliation | Sample type | Indicator Value | p. value |
|---|---|---|---|
| Bacteria-Acidobacteria-Blastocatellia-Blastocatellales-Blastocatellaceae (Subgroup 4)-Blastocatella | Soil | 0.833 | 0.005 |
| Bacteria-Acidobacteria-Blastocatellia-Blastocatellales-Blastocatellaceae (Subgroup 4)-Multi-affiliation | Soil | 0.925 | 0.005 |
| Bacteria-Bacteroidetes-Sphingobacteriia-Sphingobacteriales-Saprospiraceae-Aureispira | Soil | 0.752 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Xanthomonadales-Solimonadaceae-Multi-affiliation | Soil | 0.698 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Myxococcales-Nannocystaceae-unknown genus | Soil | 0.743 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Myxococcales-Polyangiaceae-unknown genus | Soil | 0.842 | 0.005 |
| Bacteria-Fibrobacteres-Fibrobacteria-Fibrobacterales-Fibrobacteraceae-possible genus 04 | Soil | 0.898 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Myxococcales-bacteriap25-unknown genus | Soil | 0.803 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Bradymonadales-unknown family-unknown genus | Soil | 0.904 | 0.005 |
| Bacteria-Acidobacteria-Holophagae-TPD-58-unknown family-unknown genus | Soil | 0.832 | 0.005 |
| Bacteria-Elusimicrobia-Elusimicrobia-Lineage IV-unknown family-unknown genus | Soil | 0.800 | 0.005 |
| Bacteria-Proteobacteria-ARKICE-90-unknown order-unknown family-unknown genus | Soil | 0.809 | 0.005 |
| Bacteria-Acidobacteria-Subgroup 25-unknown order-unknown family-unknown genus | Soil | 0.856 | 0.005 |
| Archaea-Euryarchaeota-Methanobacteria-Methanobacteriales-Methanobacteriaceae-Methanobrevibacter | Soil+Sludge | 0.866 | 0.005 |
| Archaea-Euryarchaeota-Methanobacteria-Methanobacteriales-Methanobacteriaceae-Multi-affiliation | Soil+Sludge | 1,000 | 0.005 |
| Archaea-Euryarchaeota-Methanomicrobia-Methanomicrobiales-Methanocorpusculaceae-Methanocorpusculum | Soil+Sludge | 0.979 | 0.005 |
| Archaea-Euryarchaeota-Methanomicrobia-Methanomicrobiales-Methanomicrobiaceae-Methanoculleus | Soil+Sludge | 0.707 | 0.005 |
| Archaea-Euryarchaeota-Methanomicrobia-Methanomicrobiales-Methanoregulaceae-Methanolinea | Soil+Sludge | 0.816 | 0.005 |
| Archaea-Euryarchaeota-Methanomicrobia-Methanosarcinales-Methanosaetaceae-Methanosaeta | Soil+Sludge | 0.866 | 0.005 |
| Archaea-Euryarchaeota-Methanomicrobia-Methanosarcinales-Methanosarcinaceae-Methanosarcina | Soil+Sludge | 0.977 | 0.005 |
| Archaea-Euryarchaeota-Methanomicrobia-Multi-affiliation-Multi-affiliation-Multi-affiliation | Soil+Sludge | 1,000 | 0.005 |
| Archaea-Multi-affiliation-Multi-affiliation-Multi-affiliation-Multi-affiliation-Multi-affiliation | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Acidobacteria-Holophagae-Holophagales-Holophagaceae-Geothrix | Soil+Sludge | 0.681 | 0.005 |
| Bacteria-Actinobacteria-Acidimicrobiia-Acidimicrobiales-Acidimicrobiales Incertae Sedis-Candidatus Microthrix | Soil+Sludge | 0.918 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Actinomycetales-Actinomycetaceae-Actinomyces | Soil+Sludge | 0.979 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Corynebacteriales-Dietziaceae-Dietzia | Soil+Sludge | 0.957 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Corynebacteriales-Nocardiaceae-Gordonia | Soil+Sludge | 0.996 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Frankiales-Cryptosporangiaceae-Fodinicola | Soil+Sludge | 0.997 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Micrococcales-Dermatophilaceae-Kineosphaera | Soil+Sludge | 0.842 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Micrococcales-Dermatophilaceae-unknown genus | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Micrococcales-Intrasporangiaceae-Tetrasphaera | Soil+Sludge | 0.913 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-PeM15-unknown family-unknown genus | Soil+Sludge | 0.998 | 0.005 |
| Bacteria-Actinobacteria-Actinobacteria-Propionibacteriales-Propionibacteriaceae-unknown genus | Soil+Sludge | 0.979 | 0.005 |
| Bacteria-Actinobacteria-Thermoleophilia-Solirubrobacterales-TM146-unknown genus | Soil+Sludge | 0.972 | 0.005 |
| Bacteria-Aminicenantes-unknown class-unknown order-unknown family-unknown genus | Soil+Sludge | 0.913 | 0.005 |
| Bacteria-Atribacteria-Atribacteria Incertae Sedis-unknown order-unknown family-Candidatus Caldatribacterium | Soil+Sludge | 0.791 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidetes vadinHA17-unknown order-unknown family-unknown genus | Soil+Sludge | 1.000 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Bacteroidales UCG-001-unknown genus | Soil+Sludge | 0.842 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-GZKB124-unknown genus | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-M2PB4-65 termite group-unknown genus | Soil+Sludge | 0.791 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Marinilabiaceae-Ruminofilibacter | Soil+Sludge | 0.705 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Marinilabiaceae-unknown genus | Soil+Sludge | 0.736 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Porphyromonadaceae-Macellibacteroides | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Porphyromonadaceae-Multi-affiliation | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Porphyromonadaceae-Petrimonas | Soil+Sludge | 0.935 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Porphyromonadaceae-unknown genus | Soil+Sludge | 0.789 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Prolixibacteraceae-Prolixibacter | Soil+Sludge | 0.645 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Rikenellaceae-Anaerocella | Soil+Sludge | 0.645 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Rikenellaceae-Blvii28 wastewater-sludge group | Soil+Sludge | 0.841 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Rikenellaceae-Multi-affiliation | Soil+Sludge | 0.890 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Rikenellaceae-S50 wastewater-sludge group | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidales-Rikenellaceae-vadinBC27 wastewater-sludge group | Soil+Sludge | 1.000 | 0.005 |
| Bacteria-Bacteroidetes-Bacteroidia-Bacteroidia Incertae Sedis-Draconibacteriaceae-unknown genus | Soil+Sludge | 0.957 | 0.005 |
| Bacteria-Bacteroidetes-Flavobacteriia-Flavobacteriales-Flavobacteriaceae-unknown genus | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Bacteroidetes-Multi-affiliation-Multi-affiliation-Multi-affiliation-Multi-affiliation | Soil+Sludge | 0.935 | 0.005 |
| Bacteria-Bacteroidetes-Sphingobacteriia-Sphingobacteriales-Lentimicrobiaceae-Lentimicrobium | Soil+Sludge | 0.677 | 0.005 |
| Bacteria-Bacteroidetes-Sphingobacteriia-Sphingobacteriales-Sphingobacteriaceae-Multi-affiliation | Soil+Sludge | 0.705 | 0.005 |
| Bacteria-Chloroflexi-43831-unknown order-unknown family-unknown genus | Soil+Sludge | 0.935 | 0.005 |
| Bacteria-Chloroflexi-Anaerolineae-Anaerolineales-Anaerolineaceae-Leptolinea | Soil+Sludge | 0.842 | 0.005 |
| Bacteria-Chloroflexi-Anaerolineae-Anaerolineales-Anaerolineaceae-Longilinea | Soil+Sludge | 0.791 | 0.005 |
| Bacteria-Chloroflexi-Anaerolineae-Anaerolineales-Anaerolineaceae-Multi-affiliation | Soil+Sludge | 0.957 | 0.005 |
| Bacteria-Cloacimonetes-Cloacimonetes Incertae Sedis-unknown order-unknown family-Candidatus Cloacamonas | Soil+Sludge | 0.866 | 0.005 |
| Bacteria-Cloacimonetes-Multi-affiliation-Multi-affiliation-Multi-affiliation-Multi-affiliation | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Cloacimonetes-W5-unknown order-unknown family-unknown genus | Soil+Sludge | 0.889 | 0.005 |
| Bacteria-Firmicutes-Bacilli-Lactobacillales-Carnobacteriaceae-Multi-affiliation | Soil+Sludge | 0.838 | 0.005 |

| | | | |
|---|---|---|---|
| Bacteria-Firmicutes-Clostridia-Clostridiales-Caldicoprobacteraceae-Caldicoprobacter | Soil+Sludge | 0.890 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Christensenellaceae-Christensenellaceae R-7 group | Soil+Sludge | 0.935 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Clostridiaceae 1-unknown genus | Soil+Sludge | 0.866 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Clostridiales vadinBB60 group-unknown genus | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Family XIII-Family XIII UCG-002 | Soil+Sludge | 0.812 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Family XIII-unknown genus | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Family XI-Sedimentibacter | Soil+Sludge | 1.000 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Gracilibacteraceae-Gracilibacter | Soil+Sludge | 0.912 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Gracilibacteraceae-Multi-affiliation | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Heliobacteriaceae-Hydrogenispora | Soil+Sludge | 0.935 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Lachnospiraceae-unknown genus | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Multi-affiliation-Multi-affiliation | Soil+Sludge | 0.996 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Peptococcaceae-Pelotomaculum | Soil+Sludge | 0.913 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Peptostreptococcaceae-Multi-affiliation | Soil+Sludge | 0.995 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Peptostreptococcaceae-Proteocatella | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Ruminococcaceae-Ruminiclostridium | Soil+Sludge | 0.785 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Ruminococcaceae-Ruminiclostridium 9 | Soil+Sludge | 0.791 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Ruminococcaceae-Ruminococcaceae NK4A214 group | Soil+Sludge | 0.677 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Ruminococcaceae-unknown genus | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Syntrophomonadaceae-Multi-affiliation | Soil+Sludge | 0.999 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Syntrophomonadaceae-Syntrophomonas | Soil+Sludge | 1.000 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Clostridiales-Syntrophomonadaceae-unknown genus | Soil+Sludge | 0.736 | 0.005 |
| Bacteria-Firmicutes-Clostridia-Thermoanaerobacterales-Thermoanaerobacteraceae-Gelria | Soil+Sludge | 0.978 | 0.005 |
| Bacteria-Firmicutes-Negativicutes-Selenomonadales-Acidaminococcaceae-unknown genus | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Firmicutes-Negativicutes-Selenomonadales-Veillonellaceae-unknown genus | Soil+Sludge | 0.736 | 0.005 |
| Bacteria-Ignavibacteriae-Ignavibacteria-Ignavibacteriales-PHOS-HE36-unknown genus | Soil+Sludge | 0.887 | 0.005 |
| Bacteria-Proteobacteria-Alphaproteobacteria-Rhizobiales-alphaI cluster-unknown genus | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Proteobacteria-Alphaproteobacteria-Rhodospirillales-Acetobacteraceae-Stella | Soil+Sludge | 0.816 | 0.005 |
| Bacteria-Proteobacteria-Alphaproteobacteria-Rhodospirillales-Rhodospirillaceae-Defluviicoccus | Soil+Sludge | 0.917 | 0.005 |
| Bacteria-Proteobacteria-Alphaproteobacteria-Rickettsiales-Rickettsiaceae-unknown genus | Soil+Sludge | 0.670 | 0.005 |
| Bacteria-Proteobacteria-Alphaproteobacteria-Sphingomonadales-Sphingomonadaceae-unknown genus | Soil+Sludge | 0.842 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Burkholderiales-Burkholderiaceae-Lautropia | Soil+Sludge | 0.933 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Burkholderiales-Burkholderiaceae-Limnobacter | Soil+Sludge | 0.833 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Burkholderiales-Comamonadaceae-Leptothrix | Soil+Sludge | 0.968 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Hydrogenophilales-Hydrogenophilaceae-Thiobacillus | Soil+Sludge | 0.764 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Hydrogenophilales-Hydrogenophilaceae-unknown genus | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Nitrosomonadales-Gallionellaceae-Candidatus Nitrotoga | Soil+Sludge | 0.657 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Rhodocyclales-Rhodocyclaceae-Azoarcus | Soil+Sludge | 0.825 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Rhodocyclales-Rhodocyclaceae-Candidatus Accumulibacter | Soil+Sludge | 0.943 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Rhodocyclales-Rhodocyclaceae-Sulfuritalea | Soil+Sludge | 0.612 | 0.005 |
| Bacteria-Proteobacteria-Betaproteobacteria-Rhodocyclales-Rhodocyclaceae-Thauera | Soil+Sludge | 0.842 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Bdellovibrionales-Bacteriovoracaceae-unknown genus | Soil+Sludge | 0.610 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Deltaproteobacteria Incertae Sedis-Syntrophorhabdaceae-Syntrophorhabdus | Soil+Sludge | 0.791 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Myxococcales-Archangiaceae-Anaeromyxobacter | Soil+Sludge | 0.645 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Oligoflexales-Oligoflexaceae-unknown genus | Soil+Sludge | 0.700 | 0.005 |
| Bacteria-Proteobacteria-Deltaproteobacteria-Syntrophobacterales-Syntrophaceae-Smithella | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Cellvibrionales-Porticoccaceae-C1-B045 | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Cellvibrionales-Spongiibacteraceae-BD1-7 clade | Soil+Sludge | 0.934 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Legionellales-Coxiellaceae-Aquicella | Soil+Sludge | 0.751 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Legionellales-Legionellaceae-Legionella | Soil+Sludge | 0.687 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Pseudomonadales-Moraxellaceae-unknown genus | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Run-SP154-unknown family-unknown genus | Soil+Sludge | 0.957 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Xanthomonadales-Xanthomonadaceae-Aquimonas | Soil+Sludge | 0.612 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Xanthomonadales-Xanthomonadaceae-Dokdonella | Soil+Sludge | 0.935 | 0.005 |
| Bacteria-Proteobacteria-Gammaproteobacteria-Xanthomonadales-Xanthomonadales Incertae Sedis-Candidatus Competibacter | Soil+Sludge | 0.831 | 0.005 |
| Bacteria-Spirochaetae-Spirochaetes-Spirochaetales-Spirochaetaceae-Sphaerochaeta | Soil+Sludge | 0.913 | 0.005 |
| Bacteria-Spirochaetae-Spirochaetes-Spirochaetales-Spirochaetaceae-Treponema 2 | Soil+Sludge | 0.707 | 0.005 |
| Bacteria-Spirochaetae-Spirochaetes-Spirochaetales-Spirochaetaceae-unknown genus | Soil+Sludge | 1.000 | 0.005 |
| Bacteria-Synergistetes-Synergistia-Synergistales-Synergistaceae-unknown genus | Soil+Sludge | 1.000 | 0.005 |
| Bacteria-Tenericutes-Mollicutes-Acholeplasmatales-Acholeplasmataceae-Acholeplasma | Soil+Sludge | 0.913 | 0.005 |
| Bacteria-Tenericutes-Mollicutes-Mollicutes Incertae Sedis-unknown family-Candidatus Phytoplasma | Soil+Sludge | 0.685 | 0.005 |
| Bacteria-Tenericutes-Mollicutes-Mycoplasmatales-Mycoplasmataceae-Candidatus Lumbricincola | Soil+Sludge | 0.730 | 0.005 |
| Bacteria-Tenericutes-Mollicutes-NB1-n-unknown family-unknown genus | Soil+Sludge | 0.763 | 0.005 |
| Bacteria-Thermotogae-Thermotogae-Kosmotogales-Kosmotogaceae-Mesotoga | Soil+Sludge | 0.913 | 0.005 |
| Bacteria-Thermotogae-Thermotogae-Petrotogales-Petrotogaceae-AUTHM297 | Soil+Sludge | 0.645 | 0.005 |
| Bacteria-Verrucomicrobia-LD1-PB3-unknown order-unknown family-unknown genus | Soil+Sludge | 0.842 | 0.005 |

*Table SI 9: Metal contents in mixtures of different treatments at the final time (mg of metal.kg$^{-1}$ of dry matter). The results were obtained by ICP analysis. Standard deviations are in parentheses.*

| Treatment | Persp--ective | Al | As | Cr | Cu | Mn | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|
| Control | 3y | 10145.53 (322.21) | 6.97 (0.59) | 17.57 (0.95) | 17.33 (0.38) | 351.30 (6.39) | lloq | 31.23 (1.55) | 69.33 (11.57) |
| | 6y | NA | NA | NA | NA | NA | NA | NA | NA |
| | 10y | 10145.53 (322.21) | 6.97 0.59) | 17.57 (0.95) | 17.33 (0.38) | 351.30 (6.39) | lloq | 31.23 (1.55) | 69.33 (11.57) |
| AD-control | 3y | 6842.60 (91.35) | 8.10 (2.15) | 20.37 (0.25) | 20.00 (0.62) | 388.83 (21.67) | lloq | 84.30 (2.05) | 103.70 (0.44) |
| | 6y | 10033.25 (105.57) | 6.56 (0.37) | 21.25 (0.07) | 22.60 (0.28) | 357.90 (16.55) | lloq | 30.40 (0.71) | 142.20 (14.28) |
| | 10y | 10705.23 (59.22) | 6.35 (1.38) | 35.27 (4.36) | 24.13 (0.86) | 348.83 (5.62) | 2.64 | 30.13 (1.82) | 171.10 4.10) |
| $AgNO_3$ | 3y | 9716.53 (651.41) | 7.61 (1.56) | 19.70 (1.30) | 18.83 (0.99) | 363.33 (10.40) | lloq | 32.15 (3.18) | 66.57 (2.37) |
| | 6y | 9796.93 (62.83) | 6.10 (1.94) | 16.30 (0.53) | 18.77 (0.71) | 365.17 (7.14) | lloq | 30.77 (3.18) | 61.23 (2.11) |
| | 10y | 10661.33 (796.66) | 6.30 (1.00) | 24.70 (1.61) | 18.07 (0.51) | 376.80 (31.53) | 0.04 (0.07) | 32.33 (1.60) | 76.00 (10.25) |
| AD-$AgNO_3$ | 3y | 6900.67 (404.71) | 6.61 (1.23) | 22.40 (0.61) | 19.63 (0.67) | 366.80 (7.09) | lloq | 36.57 (3.24) | 95.17 (3.19) |
| | 6y | 10420.30 (289.77) | 7.34 (1.19) | 22.60 (0.99) | 21.90 (0.42) | 339.60 (20.51) | lloq | 33.00 (3.68) | 129.50 (6.79) |
| | 10y | 10498.17 (398.17) | 6.37 (1.00) | 42.97 (6.35) | 23.33 (0.83) | 353.53 (3.82) | 8.74 (1.80) | 30.57 (1.46) | 170.77 (14.35) |
| Dis | 3y | 9453.30 (50.88) | 6.90 (1.06) | 19.13 (0.90) | 18.00 (0.62) | 353.97 (15.17) | lloq | 32.15 (0.35) | 64.47 (0.78) |
| | 6y | 9865.37 (870.69) | 6.78 (0.26) | 25.93 (3.65) | 17.87 (1.81) | 352.13 (24.22) | 0.42 (0.74) | 30.23 (3.67) | 61.23 (6.55) |
| | 10y | 10223.97 (656.73) | 7.66 (1.21) | 48.30 (5.60) | 18.60 (0.10) | 370.10 (13.31) | 10.30 (1.91) | 31.45 (1.91) | 67.47 (2.61) |
| AD-dis | 3y | 6978.57 (383.91) | 7.43 (1.55) | 20.40 (1.32) | 20.00 (0.66) | 388.93 (25.10) | lloq | 36.87 (1.59) | 98.90 (1.74) |
| | 6y | 10657.60 (NA) | 7.43 (NA) | 41.80 (NA) | 21.50 (NA) | 336.00 (NA) | 7.05 (NA) | 28.50 (NA) | 110.90 (NA) |
| | 10y | 10623.73 (357.46) | 6.90 (0.61) | 32.00 (6.21) | 23.03 (2.08) | 345.27 (13.66) | 1.36 (2.36) | 32.73 (2.29) | 159.70 (8.65) |
| AgNPs | 3y | 7507.77 (222.28) | 8.39 (1.25) | 21.73 (1.07) | 17.50 (1.08) | 390.17 (15.73) | lloq | 38.17 (3.61) | 68.00 (2.80) |
| | 6y | 10179.53 (991.10) | 6.77 (0.66) | 29.63 (10.70) | 17.90 (0.62) | 367.30 (41.75) | 2.12 (3.07) | 30.83 (2.61) | 61.17 (4.10) |
| | 10y | 10342.53 (414.30) | 6.84 (0.69) | 27.37 (5.48) | 17.97 (0.35) | 359.73 (3.44) | 1.85 (3.10) | 32.03 (1.42) | 66.90 (2.95) |
| AD-AgNPs | 3y | 6817.13 (82.95) | 6.95 (0.90) | 20.33 (0.84) | 20.20 (0.53) | 388.90 (11.92) | lloq | 35.13 (2.51) | 102.53 (2.21) |
| | 6y | 9744.00 (161.22) | 6.32 (0.03) | 30.35 (13.93) | 22.95 (2.19) | 340.85 (16.62) | lloq | 40.00 (6.08) | 130.20 (7.35) |
| | 10y | 10659.30 (162.04) | 6.81 (1.07) | 37.30 (4.39) | 22.90 (0.70) | 359.83 (2.31) | 5.10 (2.10) | 30.20 (1.65) | 148.03 (3.26) |

*Table SI 10: Metal contents in earthworms at the initial (T0, in grey) and final times (mg of metal.kg$^{-1}$ of dry matter). The results were obtained by ICP analysis. Standard deviations are in parentheses. Stars (*) indicate significant differences between a treatment with Ag and its corresponding control without Ag."lloq" means that the data is lower the limit of quantification.*

| Treatment | Perspective | Ag | Cd (no*) | Cu (no*) | Mn (no*) | Ni (no*) | Pb (no*) | Zn (no*) |
|---|---|---|---|---|---|---|---|---|
| T0 | 3y | 1.89 (2.65) | 1.99 (0.17) | 5.17 (1.37) | 19.01 (7.48) | 0.00 | 2.23 (0.39) | 137.94 (3.84) |
| | 6y | 0.00 | 2.30 (3.05) | 7.43 (2.16) | 8.95 (2.24) | 0.00 | 2.33 (0.40) | 126.04 (9.54) |
| | 10y | 1.89 (2.65) | 1.99 (0.17) | 5.17 (1.37) | 19.01 (7.48) | 0.00 | 2.23 (0.39) | 137.94 (3.84) |
| Control | 3y | lloq | 5.11 (0.58) | 7.87 (0.75) | 7.28 (1.83) | 0.58 (0.51) | 2.45 (0.47) | 134.60 (10.10) |
| | 6y | lloq | 4.33 (0.58) | 10.15 (2.14) | 7.95 (0.42) | lloq | 1.89 (0.4) | 130.50 (4.48) |
| | 10y | lloq | 5.11 (0.58) | 7.87 (0.75) | 7.28 (1.83) | 0.58 (0.51) | 2.45 (0.47) | 134.60 (10.10) |
| AD-control | 3y | lloq | 10.11 (4.17) | 11.38 (4.20) | 13.57 (3.40) | 0.85 (0.29) | 1.54 (0.16) | 137.25 (2.91) |
| | 6y | lloq | 6.92 (1.29) | 14.95 (1.05) | 24.88 (1.57) | 0.41 (0.08) | 1.92 (0.05) | 131.99 (0.10) |
| $AgNO_3$ | 3y | 22.19* (2.20) | 3.68 (0.47) | 5.64 (0.47) | 8.13 (1.06) | 0.10 (0.18) | 1.63 (0.41) | 133.04 (10.63) |
| | 6y | 10.63* (5.56) | 4.39 (0.54) | 6.28 (0.38) | 5.92 (1.67) | lloq | 2.53 (1.10) | 140.62 (8.07) |
| | 10y | 14.12* (3.54) | 4.26 (0.70) | 12.58 (0.31) | 5.02 (0.54) | 0.21 (0.22) | 1.83 (0.15) | 135.74 (10.71) |
| AD-$AgNO_3$ | 3y | 0.35 (0.06) | 7.50 (2.71) | 11.32 (2.32) | 16.00 (2.35) | 1.31 (0.21) | 1.43 (0.26) | 147.82 (6.06) |
| | 6y | 0.98* (0.23) | 5.23 (1.14) | 13.28 (1.52) | 18.23 (2.90) | 1.82 (0.15) | 1.67 (0.35) | 127.94 (9.14) |
| Dis | 3y | lloq | 7.14 (0.73) | 6.54 (2.20) | 7.20 (0.91) | 0.31 (0.05) | 1.39 (0.03) | 132.06 (3.80) |
| | 6y | lloq | 4.78 (2.26) | 9.65 (0.41) | 5.92 (0.62) | lloq | 2.21 (0.23) | 133.07 (5.29) |
| | 10y | lloq | 5.83 (0.77) | 7.92 (2.32) | 6.46 (0.82) | 1.17 (1.80) | 1.38 (0.31) | 140.24 (11.22) |
| AD-dis | 3y | lloq | 8.53 (1.51) | 11.06 (2.65) | 10.97 (1.96) | 0.66 (0.31) | 1.65 (0.04) | 130.64 (10.95) |
| | 6y | lloq | 6.42 (0.05) | 15.61 (3.62) | 19.81 (4.51) | 0.25 (0.35) | 2.04 (0.12) | 137.30 (7.09) |
| AgNPs | 3y | 14.97* (5.85) | 4.65 (0.36) | 5.11 (0.54) | 8.37 (2.44) | 0.17 (0.15) | 1.46 (0.34) | 137.22 (4.49) |
| | 6y | 21.32* (11.04) | 2.97 (0.41) | 6.37 (1.43) | 8.34 (2.80) | lloq | 1.83 (0.14) | 137.25 (24.12) |
| | 10y | 11.37* (0.73) | 4.15 (0.47) | 10.14 (1.94) | 5.05 (0.75) | lloq | 2.18 (0.39) | 130.88 (7.23) |
| AD-AgNPs | 3y | 0.39 (0.13) | 9.84 (0.49) | 10.58 (0.64) | 14.22 (0.96) | 1.09 (0.25) | 1.69 (0.12) | 137.48 (3.46) |
| | 6y | 0.54 (0.13) | 7.90 (0.62) | 13.44 (1.77) | 25.81 (6.03) | 0.51 (0.18) | 2.17 (0.08) | 130.72 (1.26) |

## References

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Suite aux résultats obtenus lors de cette expérimentation, de nombreuses questions restaient en suspens :

- Ces résultats sont-ils reproductibles ?
- Qu'observerait-on avec un autre type de sol ?
- Pourrait-on cibler l'observation des effets de l'Ag sur les microorganismes actifs (via les ARN) du sol et non plus la communauté totale (via l'ADN) ?
- Les activités microbiennes spécifiques comme la nitrification et la dénitrification sont-elles affectées ?
- Les 3 gènes cibles qui ne semblaient pas différentiellement régulés par l'Ag sulfuré chez *E. fetida* le sont-ils toujours à différents pas de temps et dans un autre environnement/sol ?

Ainsi, nous avons réalisé une seconde expérimentation basée sur le schéma expérimental précédent, avec quelques modifications comme l'utilisation d'une seule concentration d'argent, mais de deux sols différents, afin de préciser nos résultats précédents. Un sol provenait d'une parcelle agricole, l'autre d'un jardin de maison. Le choix de ces deux sols s'est fait en raison de leur grande différence en teneur en matière organique ainsi que leur fréquence de perturbation (l'un subissant des modifications majeures annuellement, l'autre n'étant jamais perturbé par la main de l'homme). Nous nous attendions ainsi à des communautés microbiennes significativement différentes entre ces deux sols.

Cette expérimentation fut exploitée en 2 temps différents.

Tout d'abord, nous avons travaillé sur les effets de l'argent sur la transcription des gènes *cadmium-métallothionéine*, *lysénine* et *superoxyde dismutase* chez *E. fetida*, habituellement impliqués dans les mécanismes de défense contre les métaux et/ou les stress, à divers moments

de l'exposition (1, 3 et 5 semaines). Le but était de vérifier leur implication dans le mécanisme de régulation de l'Ag au cours du temps et selon la nature du sol auquel était exposée l'espèce. La publication suivante décrit le schéma expérimental et énonce les résultats concernant les vers de terre. Cette étude nous a permis de confirmer l'hypothèse que les protéines cadmium métallothionéine (CdMT) pouvaient être impliquées dans la gestion de l'Ag sulfuré chez *E. fetida*, mais cela dépend du type de sol dans lequel ils sont. Les gènes codant pour la lysénine et la superoxyde dismutase ne sont pas influencés par la présence d'argent sulfuré dans le sol. Ceci, accompagné de l'absence d'effet de l'$Ag_2S$ sur la survie et la biomasse des vers de terre, indique que l'Ag sulfuré apporté dans l'environnement via une boue, n'engendre que peu de stress chez les vers de terre *E. fetida*, bien qu'ils bioaccumulent légèrement l'Ag sous cette forme.

# Impact of silver forms brought by sewage sludge spreading on two different soils on the test species *Eisenia fetida*.

**Pauline Courtois**[1*], Anna Grosser[3], Agnieszka Rorat[1], Sébastien Lemiere[1], Christine Lors[1,2], Anna Grobelak[3] and Franck Vandenbulcke[1]

[1] *Univ. Lille, Institut Mines-Télécom, Univ. Artois, Junia, ULR 4515 - LGCgE, Laboratoire de Génie Civil et géo-Environnement, F-59000 Lille, France*

[2] *IMT Lille Douai, Institut Mines-Télécom, Centre for Materials and Processes, F-5900 Lille, France*

[3] *Częstochowa University of Technology, Faculty of Infrastruture and Environment, Czestochowa, Poland*

Not yet submitted

**Abstract**

Due to their interesting properties, notably the biocidal properties, silver nanoparticles (AgNPs) are more and more incorporated in consumer products, which leads to release of AgNPs to the environment, especially wastewater. Silver (Ag) will be captured in sewage sludge (SS) during water treatment and since many countries recycle SS by spreading them on agricultural soils, it results in depositing Ag and its derivatives in the soil. AgNPs effects on invertebrates are rather well known. However, studies concerning the realistic mode of supply of Ag in the environment, that is to say via the supply of SS weakly concentrated in Ag sulfided, are rare. This therefore does not allow the risk to be measured. An impact of sulfided Ag on soil organisms such as earthworms could affect soil fertility and crops in the long term.

This study consisted to the exposure of *Eisenia fetida* earthworms in microcosms with Ag under different speciations for 5 weeks in two types of natural soils. AgNPs and ionic Ag ($AgNO_3$) have been added directly to the soils of some microcosms. In other microcosms, sulfided Ag (derived from same native AgNPs or $AgNO_3$) was supplied via a fermented SS in the soils. The effects of speciation of Ag, type of soil, addition of SS, could be differentiated through multiple controls. Life traits, bioaccumulation capacity and relative expression of three genes usually involved in metallic stress (*cadmium-metallothionein*, *lysenin* and *superoxide dismutase*) in *E. fetida* were analyzed. The results show that Ag, at low doses and medium term exposure, whatever its speciation, has little impact on this species. Life traits were unaffected but Ag affected the *cdmt* gene when added in its sulfide form. Although anaerobic digestion reduces the bioavailability of Ag, earthworms can still bioaccumulate it and therefore the transfer of Ag in the food chain remains a possibility.

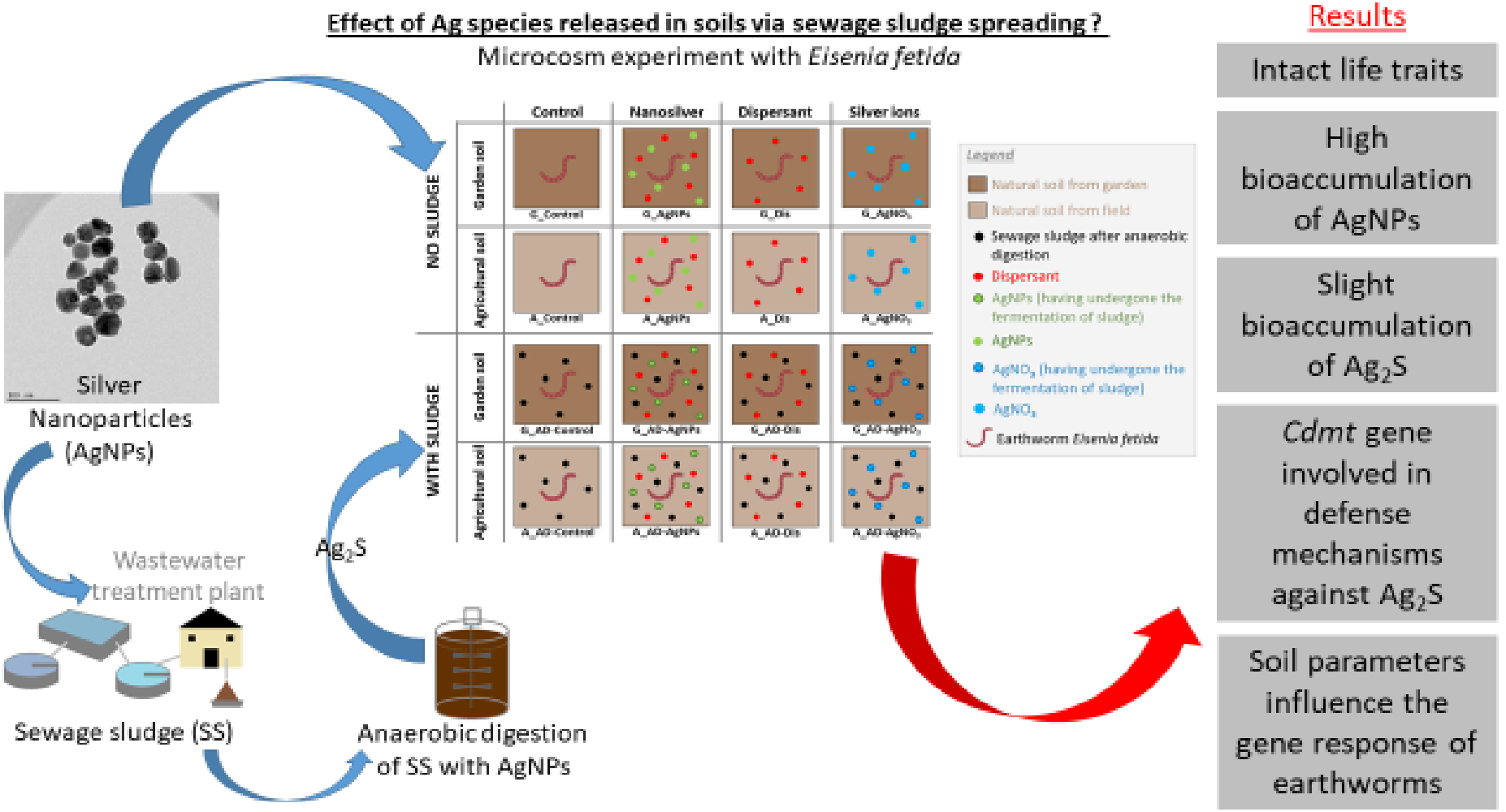
**Keywords**

Silver, speciation, earthworms, bioaccumulation, gene expression

**Highlights**

- AgNPs used leads to the contamination of soils by $Ag_2S$ via spreading of sewage sludge
- Earthworms significantly bioaccumulate sulfided Ag from sewage sludge
- Soil type can influence gene regulation in *E. fetida* facing Ag
- *cdmt* gene is up-regulated by sulfided Ag only in one type of soil

## Graphical abstract



## 1. Introduction

For three decades, more and more manufactured products, including consumer products, contain silver nanoparticles (AgNPs) (Gottschalk et al., 2013; McGillicuddy et al., 2017; Vance et al., 2015). AgNPs are incorporated for their excellent biocidal property among others (Bone et al., 2012; Maillard and Hartemann, 2013; Morones et al., 2005; Reidy et al., 2013). The life cycle of these nanofunctionalized products generates considerable releases into the environment, and in particular into wastewater (McGillicuddy et al., 2017). The efficiency of the technologies within the treatment plants makes it possible to trap in the sewage sludge (SS) most of Ag species (Kaegi et al., 2011; Tiede et al., 2010). However, since SS is a waste rich in organic matter, it is often recycled by spreading it on agricultural land (European commission, 2017). Soil is therefore one of the main places where Ag species are deposited (Massarsky et al., 2014).

Due to the amounts of Ag released and the amounts of SS produced and spread each year, it became important to accurately assess the impact of Ag species as they are brought into the soil environment. In fact, in 2008, Blaser et al. estimated that 140 tons of Ag entered agricultural soils via sewage sludge application each year in European Union, and this quantity has certainly increased since then.

Earthworms are very important to the functioning of the terrestrial ecosystem. They represent a significant biomass, they are considered as an indicator of soil quality and they participate in the digestion of organic matter and the remobilization of nutrients in the soil (Ojha and Devkota, 2014). Although many species can be used in ecotoxicology, the effect of soil contaminants is often evaluated using earthworms since a decrease in the population or the measure of individual stress markers can be informative of the stress that soil organisms are facing.

Currently, we know that AgNPs can affect the survival and the reproduction of earthworms at high doses (several hundred $mg.kg^{-1}$) (Bicho et al., 2016; Schlich et al., 2013; Shoults-Wilson

et al., 2010) and can be bioaccumulated from low doses (few tens $mg.kg^{-1}$) (Bourdineaud et al., 2019; Velicogna et al., 2017). Several metabolic changes were observed with low concentrations depending on the conditions (from 0.05 $mg.kg^{-1}$ for MT levels in LUFA 2.3 soil for example) (Curieses Silvana et al., 2017; Garcia-Velasco et al., 2017). For sulfided AgNPs, there is less data, it is certain that they are less bioavailable for earthworms, but bioaccumulation still occurs under many conditions. In conditions with natural soil and SS, bioaccumulation is significant from 70-90 $mg.kg^{-1}$ of Ag in soil (Lahive et al., 2017; Velicogna et al., 2017). Studies have shown that MTs, catalase, lysenin and other proteins appear to be involved in the defense mechanisms against Ag and sulfided Ag, however, they show different results probably due to differences in concentrations, but also to exposure matrices used.

This experiment aimed to better understand the internal defense mechanisms against Ag used by earthworms and especially to compare the results according to the speciation of Ag (sulfided or not) and the nature of the matrix of exposure. The experiment consisted of exposing earthworms *E. fetida* to two types of natural (field-collected) soils (agricultural and from garden) mixed with sewage sludge containing silver nanoparticles added before anaerobic digestion. Numerous controls with different chemical speciations and with or without SS have been carried out. The aim was to assess the effect, on earthworms, of derivatives of AgNPs in the chemical form in which Ag arrives in soils, being able to compare the effect of soil type and Ag speciation. The life traits and the relative expression of three genes involved in stress were. The bodily amounts of Ag and other metals in earthworms were also measured.

## 2. Materials and methods

### *2.1. Tested species*

Genetically identified *Eisenia fetida* earthworms (Homa et al., 2015) were obtained from a laboratory breeding facility (LGCgE, University of Lille), where they were fed with cow

manure ad libitum. Juvenil *E. fetida* were randomly selected, individually weighed and introduced into the microcosms. The earthworms weighed 245 mg on average (min: 160 mg, max: 363mg, mean standard deviation: 38 mg).

### *2.2. Soils*

Two types of soil were collected in April 2018, few days before the beginning of the experiment. They come from the Haut de France Region (France).

The "Agricultural soil" (GPS coordinates: 50°35'58.2"N 3°09'01.7"E) comes from a site that is the subject of a vegetable production certified organic agriculture since 2010, with no use of pesticides or chemical fertilizers since 10 years.

The "Garden soil" comes from a private garden in Wambrechies (same region, GPS coordinates: 50°41′7″ N–3°2′37″ E).

Both soils were slightly calcareous loamy clay on sand (soil map by DRAAF, 2013), classified as Luvisol (LV) according to WRB (World Reference Base) (Food and Agriculture Organization of the United Nations, 2015).

For both soils, the first 20 cm layer was collected and sieved at 5mm, without drying beforehand, to remove stones and roots from the soil, also to uncompress and aerate it.

Soils were characterized. The agricultural soil contained 5.09 ± 0.08 (standard deviation) mg $kg^{-1}$ As, 34.53 ± 0.87 mg $kg^{-1}$ Ba, 0.24 ± 0.02 mg $kg^{-1}$ Cd, 37.98 ± 1.88 mg $kg^{-1}$ Cr, 20.80 ± 0.22 mg $kg^{-1}$ Cu, 14.40 ± 0.18 mg $kg^{-1}$ Ni, 51.03 ± 1.78 mg $kg^{-1}$ Pb and 60.28 ± 1.00 mg $kg^{-1}$ Zn. Ag was no detectable. In the garden soil, there was 0.31 ± 0.61 mg $kg^{-1}$ Ag, 5.68 ± 0.41 mg $kg^{-1}$ As, 52.18 ± 5.20 mg $kg^{-1}$ Ba, 0.33 ± 0.09 mg $kg^{-1}$ Cd, 39.63 ± 4.97 mg $kg^{-1}$ Cr, 43.13 ± 1.24 mg $kg^{-1}$ Cu, 13.30 ± 0.36 mg $kg^{-1}$ Ni, 99.55 ± 20.02 mg $kg^{-1}$ Pb and 173.30 ± 12.85 mg $kg^{-1}$ Zn. The pH of the agricultural soil was 8.1 ± 0.1 (pH in water), whereas in the garden soil

it was 7.8 ± 0.0. The dissolved organic carbon in the agricultural soil and garden soil were respectively 27.31 ± 1.34 and 56.28 ± 2.50 $mg.L^{-1}$.

### *2.3. Sewage sludge*

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). A study by Suleiman et al. (2017) showed that this SS was a good source of nutrients for earthworms without inducing stress related to the presence of contaminants. The SS was characterized by metal contents: 11.53 ± 14.34 mg $kg^{-1}$ 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).

### *2.4. Silver species*

The used AgNPs in this study are the standard reference materials Ag NM300K from the European Commission Joint Research Centre (JRC), fully characterized (Klein et al., 2011). These nanoparticles (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 (Tween 20). The nominal size of 99% of NPs was approximately 15 nm, without coating. Transmission electron microscopy (TEM) indicated a size of 17 ± 8 nm. Smaller NPs of approximately 5 nm were also present (Mendes et al., 2015). The size distribution determinations by using zeta-sizer analysis resulted at about 100 nm (Klein et al., 2011). The commercial nanoparticle NM300K was kindly provided by Fraunhofer Institute for Molecular Biology and Applied Ecology IME

(Schmallenberg, Germany). Each bottle (lot) contains approximately 2 g of NM300K diluted in dispersant that corresponds to volume of 2 mL.

The solutions of AgNPs and Ag ions (from $AgNO_3$) were both diluted with milliQ water to obtain a Ag concentration of 2 mg $mL^{-1}$.

### *2.5. Experimental scheme*

#### *2.5.1. Batch anaerobic digestion of sewage sludge*

A batch anaerobic digestion of SS was performed in parallel in four continuous stirred-tank bioreactors at the beginning of 2018. In a 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. These bioreactors were glass vats filled with 6 L of SS, maintained under mesophilic condition, at a temperature of 37 °C with constant mixing (4 g) using mechanical stirrer (bioreactors also used by Grosser and Neczaj (2018).

After 52 days of anaerobic digestion (AD), the process had stabilized; the bioreactors were then stopped, and the digestates were centrifuged at 12,100 g for 15 minutes.

#### *2.5.2. Experimental mixtures*

Different mixtures were prepared: soil mixed with four digested SS, soil soaked with AgNPs or $AgNO_3$ or dispersant and control soil.

*Doses of Ag in SS*

The choice of Ag added in bioreactors is based on bibliographic study. Yang et al. (2012) showed that high doses of up to 40 mg $L^{-1}$ did not lead to significant differences in the

biofermentation process. Bioreactors can contain around 6 L of SS maximum. Thus, 240 mg of Ag were added in the bioreactors (AgNPs for the "AD-AgNPs" and $AgNO_3$ for the "AD-$AgNO_3$") to reach this concentration.

The concentration of AgNPs NM-300K solution was 2 mg $L^{-1}$. Thus, 120 mL of solution were added in the "AD-AgNPs" bioreactor to add 240 mg of Ag. A solution of silver nitrate was prepared at the same concentration of Ag (2 mg $L^{-1}$) and the same quantity of solution was added in the "AD-$AgNO_3$" bioreactor.

For the "AD-Dis" SS, the same quantity of dispersant contained in "AD-AgNPs" was added. Thus, 120 mL of dispersant solution was added in the "AD-Dis" bioreactor.

*Doses of SS in mixtures - Microcosms with sewage sludge named "AD-x"*

Doses of sludge introduced in microcosms was choosen according to the legislation. French regulation lays down maximum quantities to be spread over a period time. It is thus possible to apply up to 30 tons of dry matter per hectare over a period of 10 years (Circular DE / GE n ° 357 of 03/16/99, 1999), more severe than European legislation (Directive 86/278/ECC, 1986). The soil density being 1.25 g $cm^{-3}$ and the average depth of incorporation of sludge into agricultural soil is about 8 cm (typically 5 to 15 cm), the soil mass that is mixed with sludge over one hectare then corresponds to about 1000 tons (density = mass/volume). Thus, on a 10-years perspective, it is possible to apply 30 t of dry sewage sludge per 1000 t of soil i.e. 30 g of dry SS per kilogram of soil. SS contains about 85% water, so the wet SS mass allowed for spreading is close to 200 g of fresh SS per kg of soil per year. Most often, this maximum amount applicable to one decade is applied in several times. For this experiment, 60g of fresh SS per kg of fresh soil has been added. Each microcosm was then filled with 330g of this mixture.

*Doses of Ag in soil - Microcosms "AgNPs" and "$AgNO_3$"*

The addition of Ag in these microcosms depends on the estimate made on the amount of Ag remaining in the SS after the fermentation process. Tiede et al. (2010) and Kaegi et al. (2011) showed that the SS treatment allows to trap around 90% of Ag in biosolids. In our situation, it stays probably 216 mg of Ag (from the 240 mg initially added) in sludge. After one month of fermentation, there was around 900 g of fresh SS in each bioreactor. The addition of 60 g of SS per kilogram of fresh soil in "AD-AgNPs" and "AD-$AgNO_3$" microcosms, corresponded so to the addition of 14 mg $kg^{-1}$ of Ag. The same dose was brought in "AgNPs" and "$AgNO_3$" microcosms. The concentration of AgNPs NM-300K and $AgNO_3$ solutions was 2 mg $L^{-1}$ ; so 7 mL were added per kg of fresh soil.

The soil + dispersant mixtures ("Dis" microcosms) contained the same quantities of dispersant that "AgNPs" microcosms. So, 7 mL of dispersant solution were added for 1 kilogram of fresh soil.

### *2.5.3. Exposition in microcosms*

Earthworms were exposed in microcosms during 5 weeks.

The experiment included 8 types of microcosms:

Four treatments corresponded to direct exposure, without SS. Among these 4 treatments, two were controls, the "control" treatment (soil without any addition) and the "Dis" treatment (soil supplemented with the corresponding volume of dispersant). The two other treatments were the "AgNPs" treatment (soil supplemented with a solution of AgNPs provided in dispersant solution) and the "$AgNO_3$" treatment (soil supplemented with $AgNO_3$ solution).

Four of the treatments corresponded to indirect exposure, i.e., mixed with SS (named "AD-X"). In the same way, among these four treatments, two were controls, the "AD-control" treatment (soil supplied with SS digested without any addition) and the "AD-dis" treatment (soil supplied with SS digested with dispersant solution). The other two treatments contained Ag and were

the "AD-AgNPs" treatment (soil supplied with SS digested with AgNPs in dispersant) and the "AD-$AgNO_3$" treatment (soil supplied with SS digested with $AgNO_3$).

These 8 treatments were duplicated to test two different soils. The prefix G or A correspond to the type of soil (G for garden soil and A for agricultural soil).

Each of these 16 types of microcosms were made in triplicates to do a study of kinetics with 3 time points. And finally, these 48 microcosms have been made in 4 copies to have quadriplicates (Figure 1).

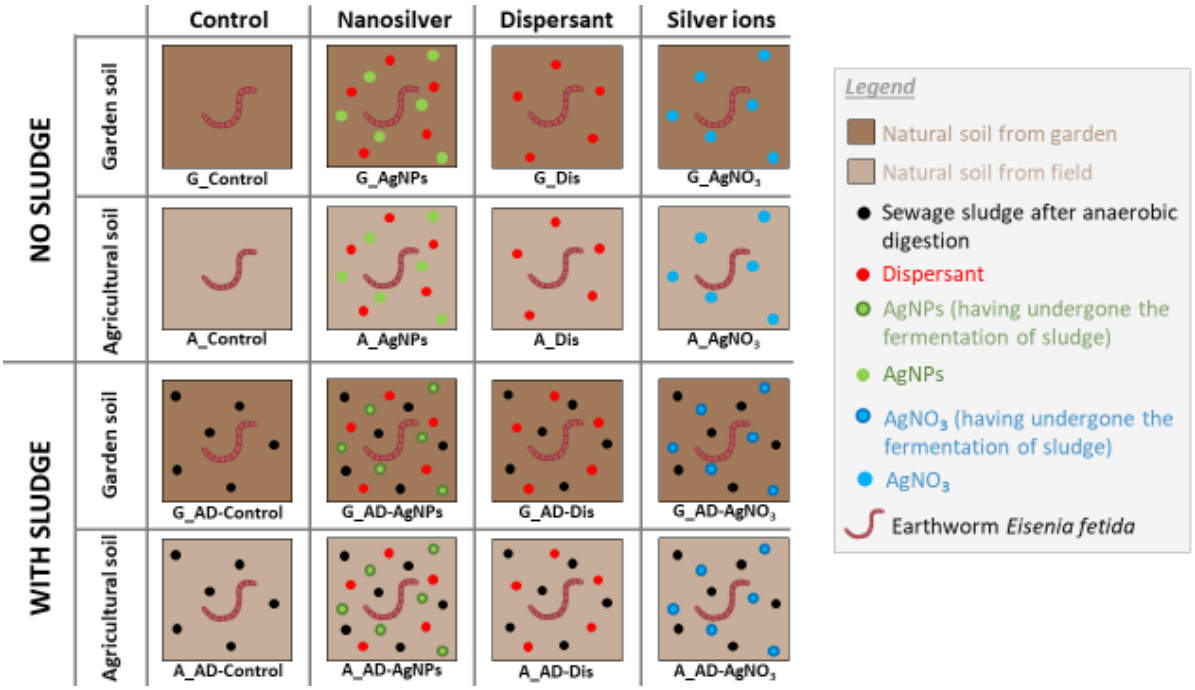



*Figure 1: Scheme of experimental design*

The mixtures were prepared as described above and distributed into plastic boxes with perforated lids (10 x 10 x 12 cm), 330 g of fresh matter per box. Six earthworms were introduced per microcosm after 1 day of incubation of the mixtures (corresponding to the time of initial depuration of the earthworms).

### *2.8. Analyses*

2.8.1. Soil mixtures and metal content

Soil samples, sludge samples and medium samples were collected at day Zero. In each microcosm, medium samples were collected also at final day. These samples were frozen and lyophilized. They were ground with a mortar and a 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.

2.8.2. Earthworms

The study of earthworms in this experiment consisted to follow the life traits (biomass, reproduction and survival) and to measure their metal content. The expression levels of some genes were also measured.

*Life traits: reproduction, survival and biomass*

The reproduction potential was estimated counting cocoons and juveniles at final time, by manual sorting of the entire soil, and observing the viability of recovered cocoons. Finally, the survival was measured counting earthworms that have survived the exposure. The biomass was followed comparing the mass of groups of organisms before and after the exposure. To erase the initial differences of weight, the value is expressed in percentage.

*Metal content*

To the day Zero of exposure, some earthworms of breading were sacrificed to measure the quantities of metal present in their organism. After the exposure, in each microcosm, some earthworms were used for the same purpose. Before the sacrifice, a depuration of 24 hours was made in order to empty the intestinal content. The organisms were sacrificed by freezing for at least 48 hours, then lyophilized for about 60 hours. The organisms were reduced to powder

using liquid nitrogen and mineralized. The mineralization is a digestion in acid medium (using $HNO_3$, $H_2SO_4$ and $HCl_4$) at high temperature. This method described in Bernard et al. (2010) was effective on reference organisms. The obtained solution was dosed by ICP-OES (Inductively coupled plasma - optical emission spectrometry) (Varian 720-ES, USA) to quantify Cd, Co, Cu, Mn, Ni, Pb, Zn and Ag.

*Gene expression levels*

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. RNA was extracted from the coelomocytes following the Tri-Reagent® protocol (Molecular Research Center, USA). Reverse transcription was performed using 0.5 µg of RNA with the Maxima H Minus Reverse Transcriptase kit (Thermo Fisher Scientific, USA). Quantitative PCR were performed with the Takyon™ SYBR® 2X qPCR Mastermix Blue no ROX (Eurogentec, Belgium). The qPCR conditions were as follows: denaturation at 95°C for 5 min, 40 cycles of amplification and extension (each cycle comprising 15 sec at 95°C, 45 sec at 60°C and 50 sec at 72°C), a melting curve step (progressive heating from 60 to 95°C), and then cooling to reach 40°C. One previously validated reference gene was used: β-actin (Forward 5'-GTACGATGAGTCCGGG-3' and 5'-GCATGTGTGTGTGGTGTC-3') (Brulle et al., 2011; Homa et al., 2015). The gene encoding β-actin (an intracellular eukaryotic protein) is the most commonly used housekeeping gene for qPCR quantification. 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 *lys* gene is required for the synthesis of the hemolytic protein LYS, which is involved in immunity and is associated 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, *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 the Stratagene Mx3000P machine (Agilent, USA) with the MxPro software. 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 $R_{treatment}/R_{associated\ treatment\ without\ silver}$.

### *2.9. Statistical analysis*

For biomass, mortality, gene expression and metal contents in earthworms, the majority of data did not follow a normal distribution and the variances were not homogeneous (Shapiro, Liliefors and Bartlett tests). Thus, Sheirer-Ray-Hare nonparametric tests and post-hoc tests based on ranks were used. For data followed a normal distribution with homogeneous variances, ANOVA tests and Tuckey post-hoc tests were used. Statistical analyses were implemented within the R programming environment (R Core Team, 2008).

## 3. Results

### *3.1. Soil mixtures*

3.3.1. Metals content and pH

At final time, the metallic composition of soil mixtures was similar in all conditions for As, Cd, Cr and Ni (Table 1). However, there were differences between the two soil types for the Cu, Pb and Zn. For Ag. There was no or little Ag in conditions without addition of Ag. In AgNPs, AD-AgNPs and AD-$AgNO_3$ conditions, there was around 9.87 ± 1.85 mg $kg^{-1}$ of Ag (dry matter), whereas in $AgNO_3$ conditions, 106.91 ± 28.19 mg $kg^{-1}$ of Ag (d. m.).

*Table 1: Metals contents in soil mixtures (mg $kg^{-1}$ of dry matter). Stars show significant differences between the condition with Ag and its respective control without Ag. "<LOQ" means below the limit of quantification.*

| Soil | Condition | Ag | As | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|
| Agricultural | Control | <LOQ | 5.08 ± 0.03 | 0.28 ± 0.16 | 38.75 ± 3.54 | 20.18 ± 0.73 | 13.60 ± 0.47 | 49.10 ± 0.99 | 60.25 ± 0.64 |
| Agricultural | Dis | <LOQ | 5.22 ± 0.17 | 0.31 ± 0.11 | 39.45 ± 4.25 | 20.05 ± 0.27 | 14.48 ± 0.46 | 53.80 ± 3.13 | 60.95 ± 1.71 |
| Agricultural | AgNPs | 12.70 ± 1.15* | 5.05 ± 0.23 | 0.31 ± 0.14 | 39.53 ± 2.87 | 21.70 ± 0.26 | 14.03 ± 0.33 | 48.88 ± 1.34 | 59.15 ± 1.48 |
| Agricultural | $AgNO_3$ | 125.98 ± 16.07** | 4.72 ± 0.17 | 0.22 ± 0.02 | 35.88 ± 1.61 | 19.35 ± 0.60 | 12.93 ± 0.35 | 49.68 ± 0.47 | 55.63 ± 1.66 |
| Agricultural | AD-control | 1.04 ± 0.30 | 4.84 ± 0.22 | 0.24 ± 0.05 | 38.60 ± 0.79 | 21.73 ± 0.29 | 13.70 ± 0.17 | 49.27 ± 1.40 | 78.87 ± 0.21 |
| Agricultural | AD-dis | <LOQ | 4.50 ± 0.26 | 0.29 ± 0.13 | 32.05 ± 0.92 | 21.63 ± 0.40 | 13.50 ± 0.25 | 49.55 ± 1.41 | 79.15 ± 2.50 |
| Agricultural | AD-AgNPs | 9.98 ± 0.37* | 4.67 ± 0.17 | 0.19 ± 0.01 | 37.33 ± 2.21 | 21.38 ± 0.96 | 13.30 ± 0.26 | 47.73 ± 1.52 | 80.00 ± 0.39 |
| Agricultural | AD-$AgNO_3$ | 9.15 ± 2.80* | 4.68 ± 0.08 | 0.10 ± 0.07 | 37.70 ± 1.20 | 21.03 ± 0.35 | 12.70 ± 0.26 | 49.03 ± 3.81 | 75.45 ± 1.96 |
| Garden | Control | <LOQ | 5.99 ± 0.99 | 0.38 ± 0.07 | 35.35 ± 2.14 | 43.45 ± 0.37 | 15.25 ± 1.53 | 90.00 ± 6.39 | 166.90 ± 11.88 |
| Garden | Dis | <LOQ | 5.54 ± 0.32 | 0.34 ± 0.09 | 39.35 ± 3.04 | 35.65 ± 1.54 | 15.48 ± 1.14 | 95.63 ± 9.50 | 170.28 ± 17.95 |
| Garden | AgNPs | 9.16 ± 1.93* | 5.85 ± 0.78 | 0.30 ± 0.04 | 38.20 ± 2.88 | 39.93 ± 0.71 | 13.45 ± 0.60 | 84.60 ± 3.57 | 140.60 ± 3.47 |
| Garden | $AgNO_3$ | 87.85 ± 25.04** | 4.98 ± 0.36 | 0.20 ± 0.03 | 33.40 ± 3.25 | 36.48 ± 0.78 | 12.70 ± 0.75 | 79.73 ± 2.38 | 126.53 ± 2.16 |
| Garden | AD-control | 2.31 ± 0.57 | 5.13 ± 0.26 | 0.30 ± 0.02 | 35.58 ± 2.14 | 43.90 ± 0.70 | 13.95 ± 0.92 | 83.90 ± 3.25 | 176.57 ± 6.41 |
| Garden | AD-dis | 0.07 ± 0.14 | 5.26 ± 0.45 | 0.34 ± 0.04 | 32.70 ± 7.17 | 42.75 ± 0.62 | 13.48 ± 1.68 | 85.38 ± 8.54 | 180.98 ± 10.48 |
| Garden | AD-AgNPs | 9.85 ± 0.50* | 5.09 ± 0.16 | 0.25 ± 0.03 | 37.55 ± 5.11 | 37.43 ± 0.40 | 12.98 ± 0.56 | 79.40 ± 5.29 | 167.55 ± 4.14 |
| Garden | AD-$AgNO_3$ | 8.60 ± 0.64* | 4.99 ± 0.67 | 0.11 ± 0.13 | 37.78 ± 2.83 | 34.30 ± 0.57 | 12.05 ± 0.79 | 78.93 ± 9.42 | 143.08 ± 14.28 |

The pH measurements (in water) showed that the addition of sludge slightly acidified the media. pH in soils without SS were 8.1 ± 0.1 and 7.8 ± 0.0 in agricultural and garden soils,

whereas pH in soils with SS were 6.7 ± 0.2 and 6.9 ± 0.1 in agricultural AD microcosms and garden AD microcosms.

### *3.2. Earthworms*

3.2.1. Life traits: survival and biomass

The survival rates were not significantly different between all conditions, with or without Ag, with or without SS, whatever the type of soil (Table 2). Indeed, in some conditions, like $AgNO_3$ in agricultural soil, the standard deviation was very high because 2 of the 4 replicate microcosms showed important mortality while in the other 2, the mortality was very low.

*Table 2: Percentage of survival of groups of earthworms at final time (35 days of exposure) with standard deviation. Due to high standard deviation, there was no significant differences between all treatments, with or without Ag, with or without sewage sludge, whatever the type of soil.*

| | Agricultural | Garden |
|---|---|---|
| AD-AgNPs | 75.00 ± 21.52 | 91.67 ± 9.62 |
| AD-dis | 87.50 ± 15.96 | 91.67 ± 16.67 |
| AD-$AgNO_3$ | 66.67 ± 23.57 | 91.67 ± 16.67 |
| AD-control | 77.78 ± 9.62 | 100.00 ± 0.00 |
| AgNPs | 100.00 ± 0.00 | 95.83 ± 8.33 |
| Dis | 100.00 ± 0.00 | 95.83 ± 8.33 |
| $AgNO_3$ | 54.17 ± 39.38 | 87.50 ± 8.33 |
| Control | 91.67 ± 9.62 | 91.67 ± 9.62 |

Ag did not impact on the biomass of earthworms since no significant differences in biomass was observed between a treatment with Ag (with or without SS) and its control without Ag (Figure 2). The type of soil did not significant affect the biomass. However, the weight loss in the microcosms without SS, compared to the weight gain in the microcosms with SS, showed that the two soils without the addition of SS caused starvation in the earthworms (significant from the 3$^{rd}$ week), contrary to the beneficial effect of adding SS.

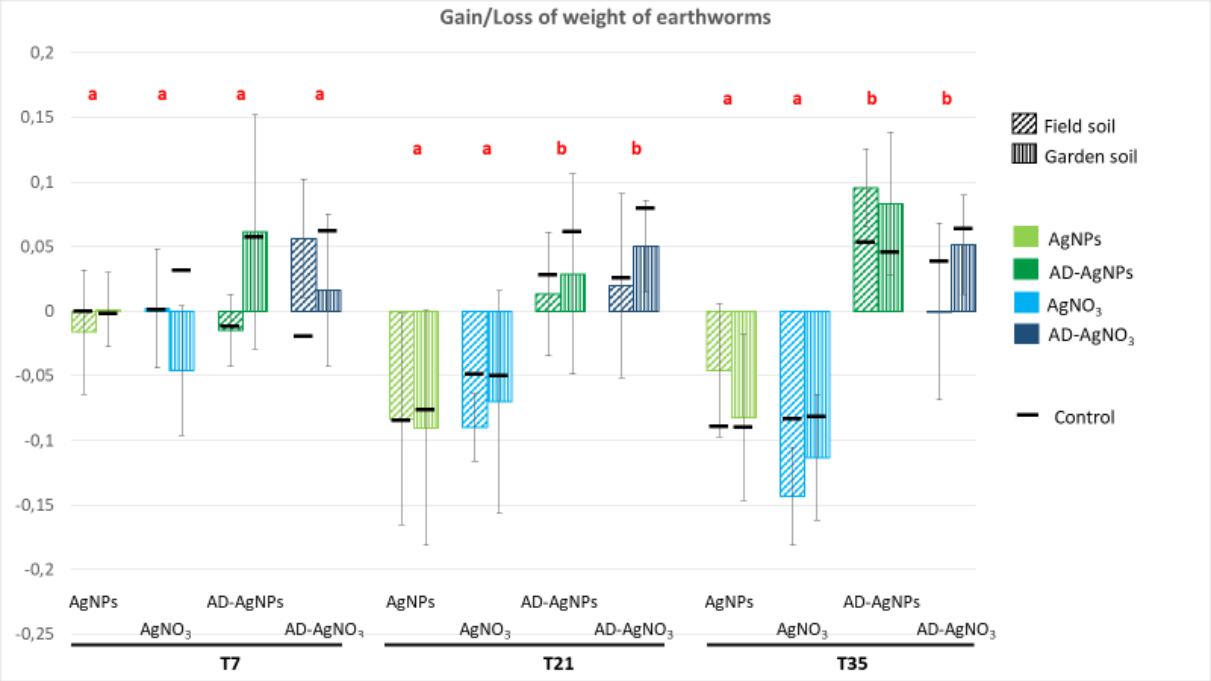



*Figure 2: Percentages of weight gain or loss in the groups of earthworms in the microcosms between the beginning and end of the experiment (percentages are reported to compensate for differences in initial weight). The black dashes correspond to the mean earthworm biomass under control conditions (dispersant for AgNPs and control for $AgNO_3$). T7, T21 and T35 correspond to 7, 21 and 35 days after the beginning of exposure. Letters show the significant statistical differences between treatments, at a given time point, regardless of the type of soil. No significant differences between time points for a same treatment with the same soil. No significant differences between the 2 soils for a same treatment at a given time. No significant differences between one condition (at a given time point and with one soil) and its control without silver (represented by black dash).*

### 3.2.2. Metals bioaccumulation

Earthworms accumulated significantly Ag in all silver treatments at the final time (Figure 3). Ag was more accumulated without SS, and this occurred very quickly. There were no differences of Ag accumulation between the two soils.

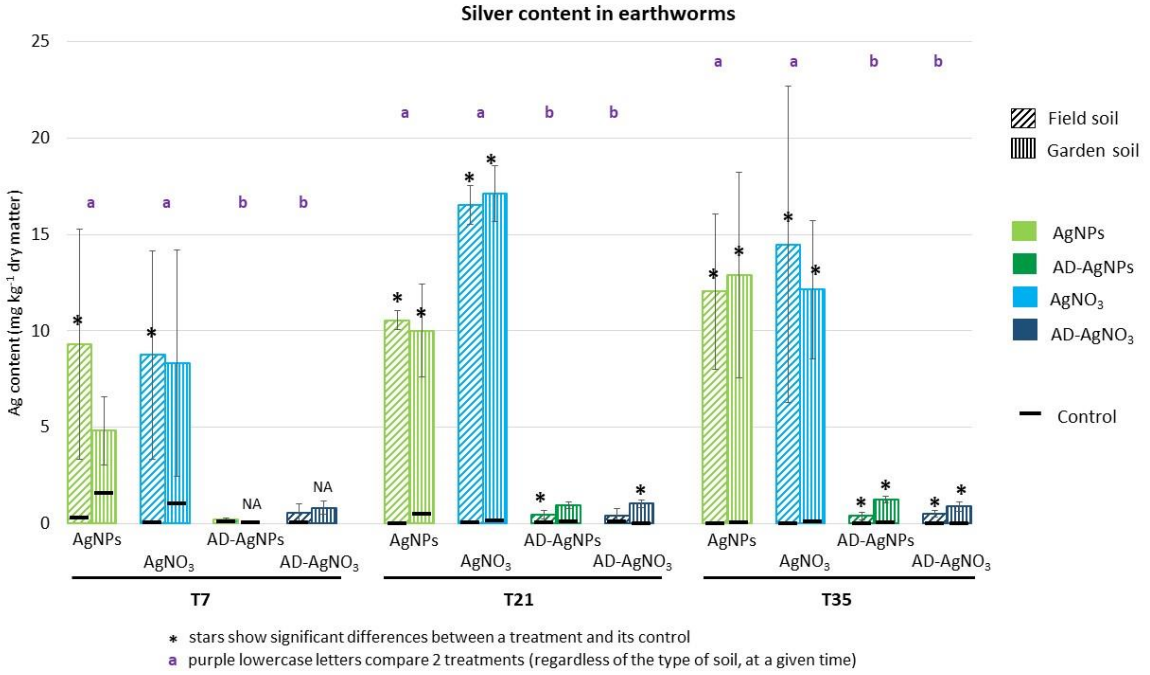



*Figure 3: Silver content in earthworms (mg $kg^{-1}$ of dry matter). The black dashes correspond to the mean content in Ag in control conditions (dispersant for AgNPs and control for $AgNO_3$). T7, T21 and T35 correspond to 7, 21 and 35 days after the beginning of exposure. Letters show the significant statistical differences between treatments, at a given time point, regardless of the type of soil. No significant differences between time points for a same treatment with the same soil. Stars show the significant differences between one condition (at a given time point and with one soil) and its control without silver (represented by black dash). NA corresponded to no available data.*

The earthworms bioaccumulated Cd independently of the type of soil but, at final time, Cd bioaccumulation was higher in earthworms exposed to SS compared to earthworms in microcosms without SS (Table 3). Silver did not influence the Cd accumulation. The bioaccumulation of Ni is quite similar than Cd: silver did not influence the Ni accumulation but earthworms bioaccumulate more Ni when they are in SS microcosms, and this difference between with and without SS is more marked in agricultural soil compared to garden soil (Table 3). For Pb, some differences of bioaccumulation can be seen between conditions with and without SS.

For As, Cu, Cr and Zn, earthworms bioaccumulated these metals independently of the type of soils and the presence / absence of SS or presence / absence of Ag.

*Table 2: Metal contents in earthworms (mg $kg^{-1}$ of dry matter) at final time (T35). "<LOQ" means below the limit of quantification.*

| Soil | Condition | As | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|
| | T0 | 10.89 ± 3.27 | 2.29 ± 0.70 | 0.21 ± 0.15 | 14.45 ± 0.91 | 0.24 ± 0.13 | 0.71 ± 0.40 | 101.16 ± 5.37 |
| Agricultural | control | 27.61 ± 13.95 | 4.88 ± 1.03 | 0.28 ± 0.09 | 11.82 ± 2.05 | 0.41 ± 0.14 | 0.12 ± 0.18 | 111.16 ± 4.43 |
| Agricultural | dis | 29.61 ± 7.82 | 5.07 ± 1.11 | 0.18 ± 0.03 | 16.14 ± 5.22 | 0.32 ± 0.09 | <LOQ | 112.93 ± 8.81 |
| Agricultural | AgNPs | 26.439 ± 13.22 | 3.99 ± 0.95 | 0.15 ± 0.02 | 12.00 ± 2.00 | 0.32 ± 0.07 | 0.06 ± 0.12 | 116.43 ± 7.23 |
| Agricultural | $AgNO_3$ | 50.35 ± 46.19 | 4.37 ± 1.90 | 0.20 ± 0.17 | 15.15 ± 6.82 | 0.60 ± 0.12 | <LOQ | 126.80 ± 6.41 |
| Agricultural | AD-control | 28.11 ± 8.83 | 7.47 ± 0.25 | 0.47 ± 0.52 | 15.72 ± 5.62 | 1.92 ± 1.33 | 0.39 ± 0.56 | 108.56 ± 13.32 |
| Agricultural | AD-dis | 19.53 ± 6.90 | 7.98 ± 1.68 | 0.20 ± 0.19 | 15.67 ± 2.22 | 1.06 ± 0.22 | 0.20 ± 0.37 | 107.10 ± 11.24 |
| Agricultural | AD-AgNPs | 12.02 ± 2.42 | 9.83 ± 1.60 | 0.28 ± 0.09 | 15.15 ± 1.31 | 1.90 ± 0.40 | 0.16 ± 0.11 | 116.25 ± 8.31 |
| Agricultural | AD-$AgNO_3$ | 11.39 ± 1.72 | 9.18 ± 2.56 | 0.08 ± 0.13 | 13.56 ± 1.93 | 1.05 ± 0.18 | 0.05 ± 0.05 | 104.25 ± 8.11 |
| Garden | control | 18.98 ± 13.10 | 3.96 ± 0.59 | 0.07 ± 0.05 | 14.99 ± 3.64 | 0.46 ± 0.15 | 0.12 ± 0.18 | 113.22 ± 8.00 |
| Garden | dis | 20.69 ± 6.85 | 2.81 ± 0.86 | >LOQ | 9.61 ± 1.92 | 0.19 ± 0.08 | <LOQ | 98.40 ± 17.97 |
| Garden | AgNPs | 27.21 ± 15.05 | 3.00 ± 0.07 | 0.04 ± 0.08 | 11.77 ± 1.14 | 0.34 ± 0.07 | 0.15 ± 0.30 | 109.95 ± 6.61 |
| Garden | $AgNO_3$ | 21.91 ± 5.00 | 2.89 ± 0.59 | 0.20 ± 0.39 | 13.66 ± 1.34 | 0.51 ± 0.12 | <LOQ | 110.69 ± 19.32 |
| Garden | AD-control | 16.67 ± 1.91 | 5.39 ± 1.92 | 0.26 ± 0.14 | 20.99 ± 2.72 | 0.67 ± 0.13 | 0.81 ± 0.44 | 110.29 ± 8.74 |
| Garden | AD-dis | 10.37 ± 2.52 | 5.30 ± 0.36 | 0.09 ± 0.12 | 19.09 ± 2.96 | 0.68 ± 0.08 | 0.21 ± 0.35 | 109.93 ± 2.50 |
| Garden | AD-AgNPs | 22.13 ± 12.92 | 6.58 ± 1.34 | 0.23 ± 0.17 | 21.71 ± 1.41 | 0.98 ± 0.21 | 0.77 ± 0.38 | 110.73 ± 1.16 |
| Garden | AD-$AgNO_3$ | 28.06 ± 7.36 | 5.22 ± 0.77 | 0.37 ± 0.24 | 22.86 ± 6.28 | 0.64 ± 0.12 | 1.26 ± 0.55 | 111.14 ± 2.91 |

3.2.3. Gene expression levels

The relative expression of *cdmt* gene was not significantly affected in agricultural soil, whatever the duration of exposure, treatment or SS presence/absence (Figure 4). In garden soil, the same result was observed after 7 and 35 days of exposure of earthworms. However, after 21 days of exposure, some significant changes were visible for *cdmt* gene relative expression (Figure 4, graphic D). The letters indicating the results of statistical tests only compared the conditions without SS with each other, and separately the conditions with SS between them. Among conditions without SS, dis and $AgNO_3$ conditions are significantly different from control and AgNPs condition was similar from three other conditions. Thus, there was an effect of any addition that decreased the relative expression of *cdmt* gene, but Ag was not responsible. However, in conditions with SS, significant differences between AD-control and both AD-Ag conditions were highlighted. AD-dis was similar to all three other conditions. There was an overexpression of *cdmt* gene with any addition in SS-soil mix and this effect was accentuated with both types of Ag. Thus, there is an effect of Ag brought by SS, whatever the initial speciation of Ag, on relative expression of *cdmt* gene (overexpression) in garden soil, visible only after 3 weeks of exposure.

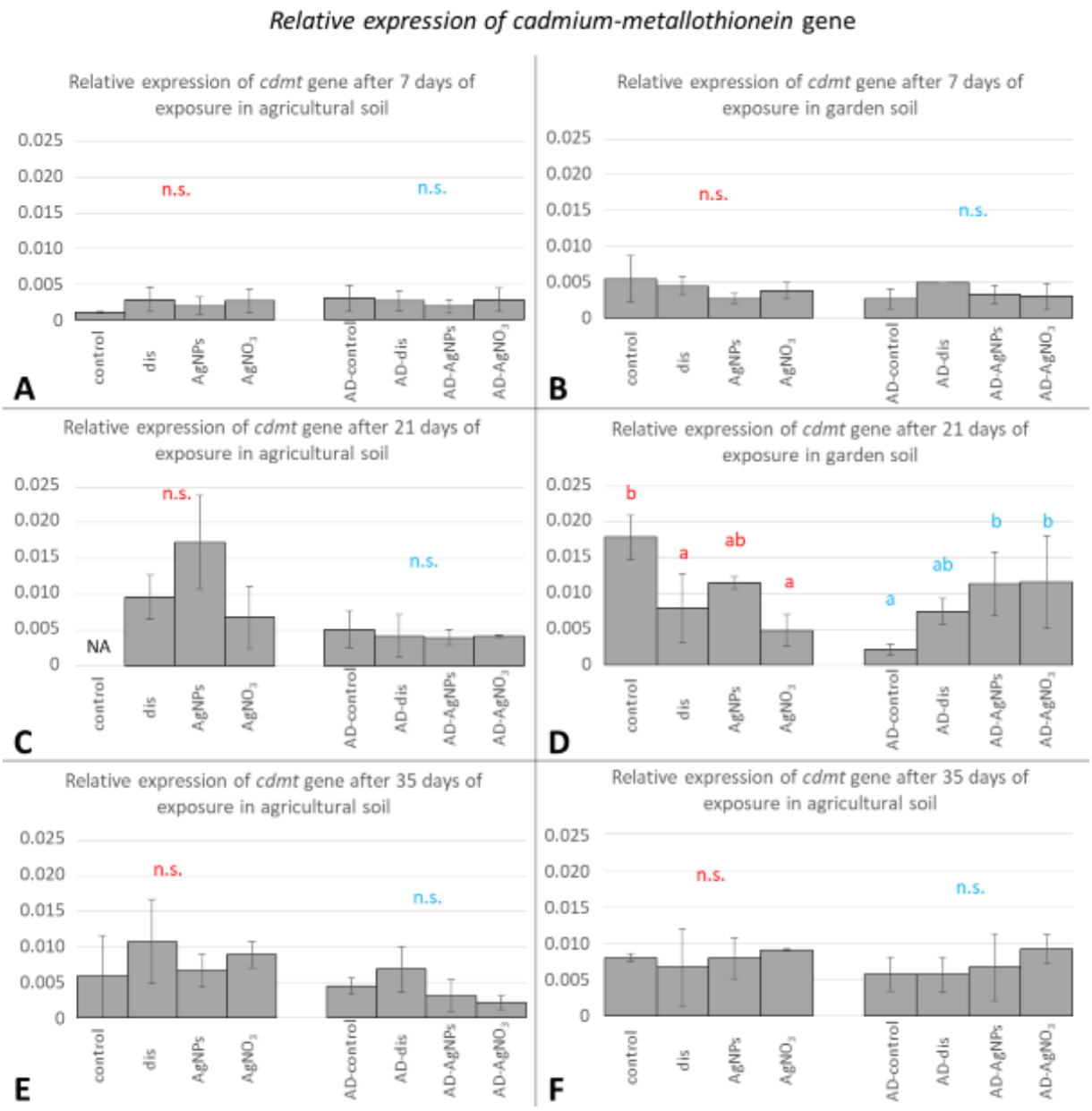



*Figure 4: Relative expression of cadmium-metallothionein gene from coelomocytes of earthworms. NA means that the data is missing. Red letters show the statistical results. "n.s." means that there was no significant difference between the 8 conditions.*

The relative expression of *sod* gene showed no difference in agricultural soil for the two first time points (Figure 5, graphics A and C), however after 5 weeks of exposure, the *sod* was downregulated in condition with SS with any additives compared to AD-control (Figure 5, graphic E). In the garden soil, there was no differences of expression of *sod* after 3 and 5 weeks

of exposure (Figure 5, graphic D and F) but at short time (7 days; Figure 5, graphic B), a significant downregulation was observed in both Ag conditions without SS, compared to control. Thus, there was an effect of Ag brought directly without SS, whatever the initial speciation of Ag, on relative expression of *sod* gene (downregulation) in garden soil, visible only after 1 week of exposure.

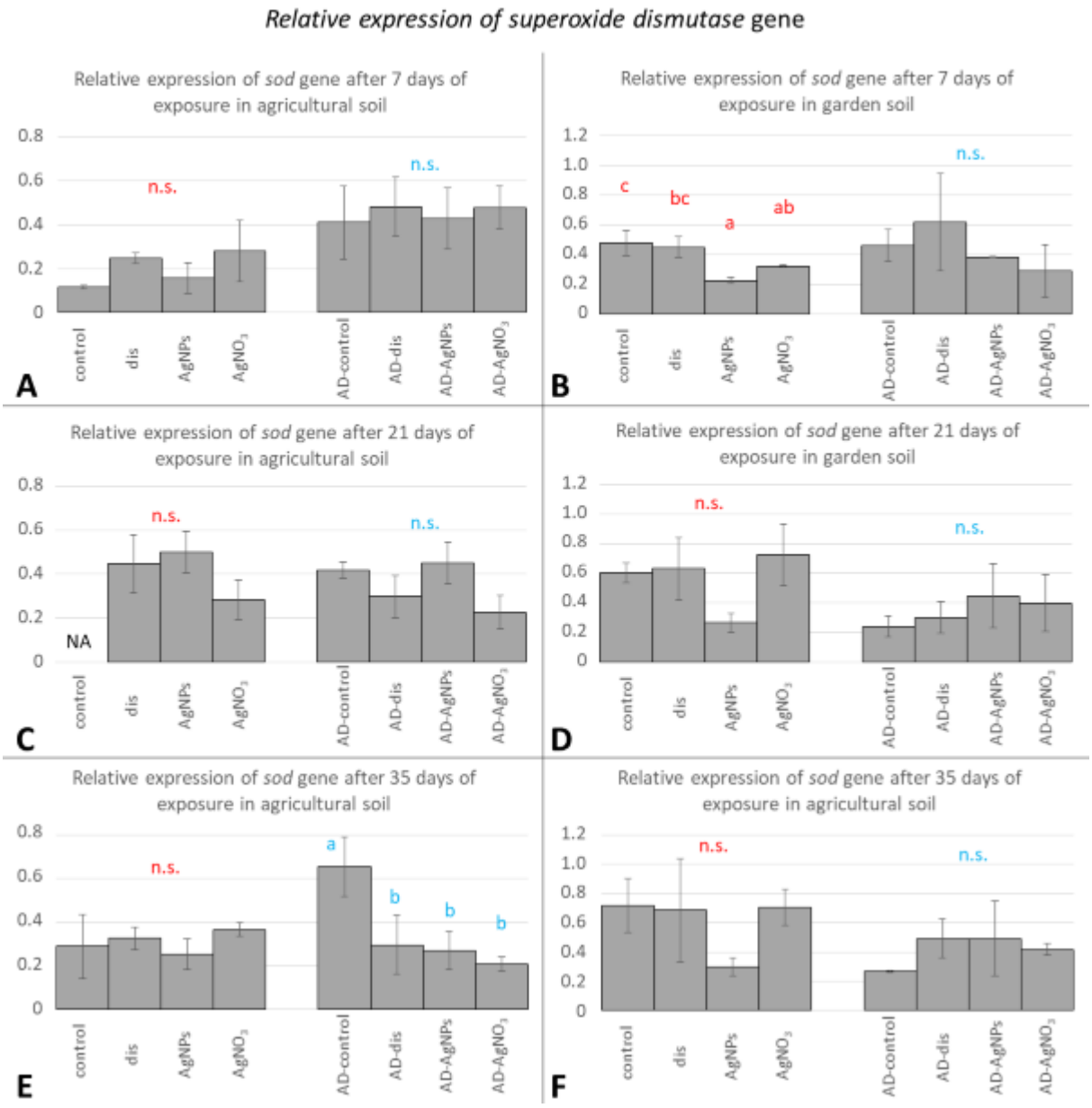



*Figure 5: Relative expression of superoxide dismutase gene from coelomocytes of earthworms. NA means that the data is missing. Red letters show the statistical results. "n.s." means that there was no significant difference between the 8 conditions.*

Finally, the relative expression of *lysenin* gene (Figure 6) varies more. The *lys* is affected by different treatments without SS in agricultural soil, at all observed time points. After 7, 21 and 35 day of exposure, the *lys* gene was significantly overexpressed in $AgNO_3$ (without SS – conditions with 10 times more of Ag compared to AgNPs) compared to control (observation

for 7 and 35 days due to the missing control at 21 days) and often also compared to other conditions without SS (dis and AgNPs) even if it is not always significant (Figure 6, graphics A, C and E). In garden soil, this phenomenon of overexpression of *lys* gene was also visible (but not significant) in $AgNO_3$ condition (without SS) but only after 7 days of exposure (Figure 6, graphic B). The *lys* gene was downregulated with any additives compared to control after 21 days of exposure in conditions without SS in garden soil (Figure 6, graphic D). Overall, under the conditions of this study, it was not possible to show the effect of Ag (even in $AgNO_3$ form) on the expression of *lys* gene since these conditions were never significantly different at the same time of the two controls (with / without dispersant).

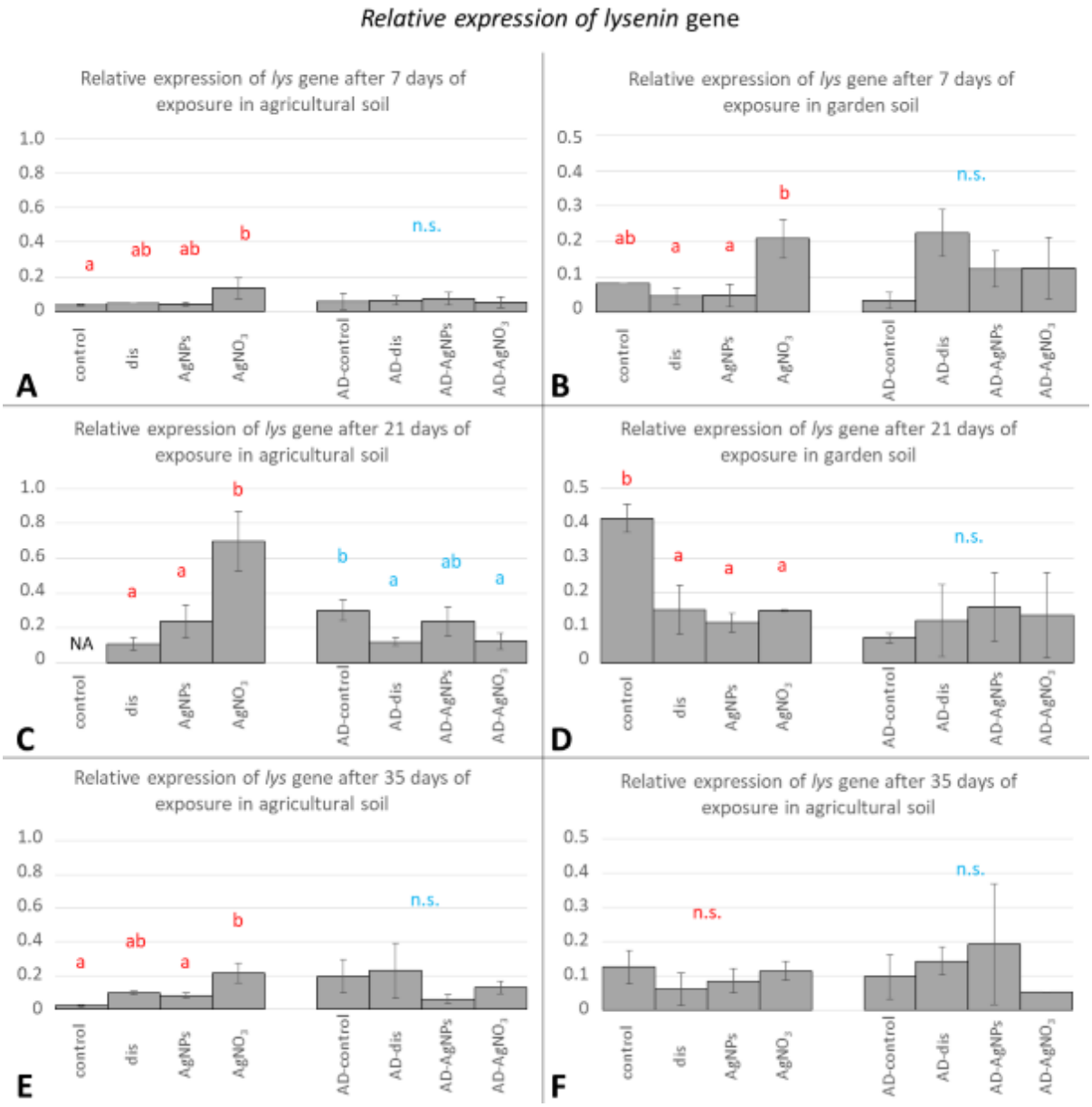



*Figure 6: Relative expression of lysenin gene from coelomocytes of earthworms. NA means that the data is missing. Red letters show the statistical results. "n.s." means that there was no significant difference between the 8 conditions.*

## 4. Discussion and conclusion

Results showed that survival and biomass of earthworms *Eisenia fetida* were unaffected by medium-term exposure (5 weeks) of approximately 10 mg $kg^{-1}$ of AgNPs, $AgNO_3$ or Ag transformed in SS. Even the concentration 10 times more concentrated of ionic Ag ($AgNO_3$)

had no impact on life traits. With low doses of Ag, these results have already been observed in several studies with different conditions (artificial or natural soil and different durations of exposure) like in Brami et al. (2017), Curieses Silvana et al. (2017), Shoults-Wilson et al. (2010) and Courtois et al. (2020a). Apparently, only very high doses of $AgNO_3$ (several hundred mg per kg of soil) can cause mortality of this species like shown in Heckmann et al. (2011), Gomes et al. (2015) and (Courtois et al., 2020b). However, the life-traits results in this present study must be observed with great care. Indeed, the differences in the quantity of organic matter between the soils (DOC in garden and agricultural soils were 56.28 ± 2.50 and 27.31 ± 1.34 mg $L^{-1}$), and especially between the conditions with and without SS (DOC in garden and agricultural supplemented with control SS were 94.70 ± 4.85 and 68.17 ± 3.68 mg $L^{-1}$), must have caused different feeding behaviors between the earthworms. The weight loss results in soils without SS showed that it would have been better to supplement the soils with organic matter in order to avoid stress in the control earthworms. However, this choice of no food supplementation was made because of a parallel study on the communities of microorganisms present in the soils, in order to preserve the communities and their potential activities as they were in the field.

However, this earthworm species is able to bioaccumulate significant amounts of Ag, up to 10-15 mg $kg^{-1}$ (dry matter) in its body and this is done very quickly (1 to 3 weeks). The bioaccumulation depends on the speciation of Ag in the soil, but apparently not on the concentration in the soil. Indeed, the earthworms have bioaccumulated around 12.10 mg $kg^{-1}$ d. m. (with a maximum bioaccumulation at 38.68 mg $kg^{-1}$ d.m.) when initial form of Ag provided was nanoparticulate or ionic (direct contamination without SS) while the intake of Ag coming from the SS, led to 10 times less bioaccumulation in mean (max 12.60 mg $kg^{-1}$). A previous study showed that AgNPs and $AgNO_3$ were both transformed in sulfided Ag during the anaerobic digestion and this Ag species was then stable during several weeks in this agricultural

soil studied here (Courtois et al., 2020a) and previous works had already shown that AgNPs were sulfided during the processes of wastewater treatment and SS remediation (Kaegi et al., 2013, 2011; Kent et al., 2014; Levard et al., 2012; Pradas del Real et al., 2016) and that they were stable (Donner et al., 2015; Lombi et al., 2013). Therefore, the bioaccumulation of Ag by earthworm depends to the speciation of Ag (sulfided or not), since sulfide Ag is more stable and releases less $Ag^+$ ions than AgNPs (Levard et al., 2013, 2011; Nowack et al., 2011). In addition, there is no difference in bioaccumulation between earthworms exposed to 10 mg $kg^{-1}$ AgNPs or 100 mg $kg^{-1}$ $AgNO_3$. These results in two different soil matrices validates the preliminary results on the subject showing that bioaccumulation depends on chemical form of Ag (Courtois et al., 2020a) and that earthworms have a capacity to regulate quantities of bioaccumulated Ag (Courtois et al., 2020b). The pH and the organic matter contents of soils are known to influence the bioavailability of metallic elements. Indeed, Ag can form complexes with humic acids making Ag less bioavailable (Cornelis et al., 2012; McKee et al., 2019) and a more acidic pH results in a higher bioavailability of Ag (Topuz and van Gestel, 2017). However, here the differences between soils do not seem to have played a significant role in the bioaccumulation of Ag by the worms. However, for the differences in bioaccumulation observed between worms exposed or not to mud, these two parameters may have contributed equally.

Care should also be taken in interpreting gene expression results since starvation stress under the SS-free conditions may have altered the earthworm metabolism and hence its response to Ag. However, we can observe, under these conditions without SS, the effect of Ag on earthworms in food stress situation. Under the conditions with SS, this stress was not present, the conditions with and without SS should therefore be compared with caution.

In previous studies on the effects of Ag on the expression of *Eisenia fetida* genes, *cdmt* gene has often been differentially regulated because of Ag presence. The *cdmt* gene was downregulated after 10 days of exposure with 2 mg $kg^{-1}$ of $AgNO_3$ (Bourdineaud et al., 2019) and this was no longer the case when there were AgNPs in the microcosms or when the concentration of $AgNO_3$ increased. Moreover, the study of Curieses Silvana et al., 2017 showed that *cdmt* was upregulated since 1 day of exposure for high concentrations of AgNPs and $AgNO_3$ (50 mg $kg^{-1}$) and since 3 days for the low concentrations (0.05 mg $kg^{-1}$). However, for 14 days of exposure, while the difference in *mt* regulation disappeared with $AgNO_3$; with both concentrations of AgNPs, there was a down-regulation. In another work, after 7 days of exposure, a dowregulation of *mt* was seen for low concentrations of AgNPs (1 and 10 mg $kg^{-1}$ d. m.), but it was not significant. Conversely, a significant overexpression was observed in the presence of high concentrations of $AgNO_3$ (Choi and Park, 2015). These authors cited three studies used artificial soil unlike the present study. This present study shown less variation in *cdmt* gene expression but this difference is not due to a lower bioavailability of Ag for earthworms in natural soil compared to artificial soil like in the Bourdineaud et al., (2019) and Choi and Park (2015) studies, the bioaccumulation was weaker than here (no measurement of bioaccumulation in Curieses Silvana et al. (2017)). In a natural soil, one study (Hayashi et al., 2013) saw the induction of the *mt* gene the first day of exposure with AgNPs but at very high concentration (500 mg $kg^{-1}$ d.m.). To compare with this study in natural soil, we could not observe the expression of genes at such a short time (1-3 days) after the start of the experiment therefore it is not possible to know if a more realistic concentration like these present conditions induced a change in the expression of *cdmt* on the first days of the exposure. However, after 7 days of exposure, no regulation of *cdmt* gene was observed in both soils. In agricultural soil, *cdmt* gene was not differentially regulated at later time points whereas after 21 days of exposure in garden soil, upregulations of *cdmt* gene appear when Ag was brought via SS, with both initial

speciation of Ag before digestion of SS. In condition without SS (as in all studies found in the literature), *cdmt* gene was downregulated with any additive (Ag but also dispersant), we cannot therefore evoke an effect of Ag. Two weeks later (after 35 days of exposure), the *cdmt* gene was again expressed in the same way under all conditions. MTs are known to be influenced by several environmental stress like starvation (Dallinger, 1996), the response observed here in conditions without SS could be related to the lack of food. Thus, in both soils, there is no visible effect of low dose of AgNPs or high dose of $AgNO_3$, directly brought in soils, on the *cdmt* gene expression. However, sulfided Ag, brought by SS, can lead to overexpression of the *cdmt* gene in certain type of soil (in this study, only garden soil), but this is not visible at all the observed time windows. Overall, taking into account all the existing data in the bibliography, even if the exposure conditions influence the results, it would seem that the *cdmt* gene can be induced during the first days or weeks following the beginning of exposure and then its relative expression returns to normal or is even inhibited, except when the concentrations are high. Other clues show that the *cdmt* gene would have a role in the management of Ag in earthworms. Indeed, in a previous experiment, it was shown that Ag was linked to a thiolated molecule inside the worm and that Ag was localized in the tissues where MT proteins are concentrated (Courtois et al., 2020b).

In addition, the *sod* gene seems that only two studies have observed the modification of the relative expression of this gene during exposure to Ag in earthworms. On artificial soil, an overexpression of *sod* has been observed in Choi and Park (2015) with a concentration of 100 mg $kg^{-1}$ (dry soil) of $AgNO_3$ while with concentrations ranging from 1 to 100 mg $kg^{-1}$ of AgNPs, the *sod* gene was not significantly overexpressed. In natural soil, RNA extraction from entire *E. fetida* earthworms showed a downregulation of the *sod* gene after 1 or 3 days of exposure to 100 and 500 mg $kg^{-1}$ of large AgNPs (30 nm) but not with small AgNPs (10 nm) or $AgNO_3$ (Tsyusko et al., 2012). In contrast, in the Hayashi et al. (2013) study with natural soil, a

concentration of 500 mg $kg^{-1}$ of AgNPs or $AgNO_3$ did not significantly change the relative expression of *sod* gene. No study has investigated the expression of *sod* gene with low Ag concentrations. In this present work, the results were different in the two soils. In agricultural soil, there was no effect of Ag at 1, 3 and 5 weeks, whereas in garden soil, after 7 days of exposure only, both forms of Ag directly applied in soil (without SS) caused significant downregulation of the *sod* gene. This downregulation was no longer visible afterwards. The observation of a downregulation of the sod gene has already been used in other contexts of contamination in *E. fetida* and had been explained by an excessive generation of radicals exceeding the capacity of elimination of the worm, thus damaging the cells (Chen et al., 2011; Jo et al., 2008). However, observations of the regulation of the sod gene after 7, 21 and 35 days of exposure cannot allow us to state that Ag, whatever its speciation causes oxidative stress in *E. fetida*. We may still have missed other regulatory changes in this gene earlier in exposure, but this remains to be investigated.

Finaly, the *lysenin* gene is not well studied in the context of exposure to Ag however one study showed that the *lys* gene was upregulated (measure after 2 h of exposure) and then down-regulated (measure after 8 and 24 h of exposure) in earthworm's coelomocytes directly exposed to low concentrations of Ag (Hayashi et al., 2015). Here, in a more realistic context, the *lys* gene was also differentially induced with Ag in al treatments. The up-regulation that can be guessed in both soils with $AgNO_3$ without SS (10 times more concentrated condition) was not significant. Thus, lysenin gene does not allow us to document the effect of exposure to Ag on *E. fetida* in a context approaching reality.

In any case, under our conditions, the Ag, directly brought without SS, affected the relative expression of *sod* gene only in garden soil at the beginning of exposure (downregulation). Sulfided Ag, brought by SS, did not impact the relative expression of *lys* or

*sod* genes but did affect the *cdmt* gene. $Ag_2S$ resulted in an upregulation of *cdmt* gene in garden soil (visible after 3 weeks of exposure).

The study of these three genes in two different soils showed that the environment influences the toxicity / stress potential of a contaminant. Animals can be more or less sensitive to a same contaminant in a same concentration depending on the composition of their environment. Indeed, whereas in a soil one can see a stress caused by the presence of Ag, reflected by up- or downregulation of certain stress genes, in another soil, this stress may not be seen or be less marked or appear at different times. The environment therefore influences the sensitivity of earthworms to a contaminant such as Ag. The verification of the evolution of Ag species over time in both soils with synchrotron could be interesting. In fact, it is possible that speciation of Ag in agricultural and garden soils evolves differently and therefore affect earthworms differently.

*Conclusion*

The results of this study showed that sulfided Ag applied to soils via SS is less bioaccumulated by soil organisms such as earthworms. Conversely, $Ag_2S$ triggered defense mechanisms involving the *cdmt* gene that were not visible with the exposure of AgNPs and $AgNO_3$ directly in the soil. To better understand the mechanisms of regulation of Ag in *E. fetida*, it would be very interesting to carry out a transcriptomic study of the genome of the earthworm, in order to compare the expression of all the genes in control condition and to identify in Ag condition all the genes involved in the defense mechanisms against Ag. Finally, to assess the real hazardous of Ag brought into the soil by sewage sludge, it is important to take into account the concentrations, the speciation of Ag as well as the composition of the soil since it has influenced the response of earthworms at the metabolic level.

The authors declare no competing financial interest.

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**References**

At the end of the manuscript

Ensuite, nous nous sommes concentrés sur l'effet de l'$Ag_2S$ apporté par la boue d'épuration sur les microorganismes peuplant deux types de sol avec des caractéristiques pédologiques très différentes (un sol agricole et un sol de jardin). Après avoir mesuré les activités potentielles de nitrification et dénitrification, nous avons cette fois observé la diversité microbienne grâce à des prélèvements d'ARN afin d'avoir une vision globale de la communauté en activité. L'article ci-dessous décrit en détails les résultats et réflexions issues de cette expérience. Ce travail nous a permis de constater que l'Ag sulfuré apporté par une boue dans un sol affecte au moins transitoirement les communautés actives de microorganismes du sol. Selon le type de sol, cette modification peut persister davantage et être encore nettement significative 5 semaines après l'ajout de boues contaminées par l'Ag dans le sol. Cette perturbation de la communauté microbienne ne semble pas avoir affecté les microorganismes impliqués dans la nitrification puisque l'activité enzymatique nitrifiante n'a pas été affectée par l'Ag sulfuré, quels que soit le sol ou le moment observé. En revanche, de manière étonnante, l'activité de dénitrification a été négativement affectée dans un des 2 sols, ce qui pourrait suffire à déstabiliser le cycle des nutriments azotés et donc la fertilité du sol.

# Differential responses of soil microbial communities in two soils submitted to sewage sludge containing silver

Pauline Courtois[1], Franck Vandenbulcke[1], Rémy Guyoneaud[2], Claire Gassie[2], Amélie Cantarel[3], Jonathan Gervaix[3], Agnieszka Rorat[1], Sébastien Lemiere[1], Anna Grosser[4], Anna Grobelak[4], Christine Lors[1] and Éléonore Attard[2]

[1] *Univ. Lille, IMT Lille Douai, Univ. Artois, Yncrea Hauts-de-France, ULR4515 - LGCgE, Laboratoire de Génie Civil et géo-Environnement, F-59000 Lille, France*

[2] *Université de Pau et des Pays de l'Adour, E2S UPPA, CNRS, UMR IPREM 5254, Environmental Microbiology, 64000, Pau, France*

[3] *Univ Lyon, Université Claude Bernard Lyon 1, CNRS, INRAE, UMR5557 LEM, F-69622 Villeurbanne, France*

[4] *Częstochowa University of Technology, Faculty of Infrastruture and Environment, Czestochowa, Poland*

Not yet submitted

**Abstract**

Silver nanoparticles (AgNPs) are used in many consumer products notably for their antimicrobial properties, among others. Thereby, AgNPs are released in wastewater and concentrated in sewage sludge (SS) where silver (Ag) is mainly in sulfided form following chemical transformations. Due to SS spreading on agricultural lands and the lack of knowledge of effects of Ag sulfide ($Ag_2S$) on microbial communities in real conditions, it was necessary to study the effects of $Ag_2S$, brought by SS in soil, on diversity and activities of soil microflora, over time. This study consisted of monitoring soil microbial communities during 5 weeks after mixing of 2 different natural soil (agricultural or from garden) with SS contaminated by two forms of Ag (AgNPs or $AgNO_3$) before anaerobic digestion to obtain $Ag_2S$. Three different controls allowed comparisons: soil only and soil mixed with SS containing dispersant (used to solubilize AgNPs) or no additive. Diversity of active soil microbial communities via Miseq sequencing of the total RNA 16S and soil microbial functioning via measurement of the nitrifying enzyme activity (NEA) and denitrifying enzyme activity (DEA) were assessed. The results showed that $Ag_2S$, provided by SS in soil, did not affect the NEA in both soils. The DEA was not affected also in agricultural soil but $Ag_2S$ prevented the increase of DEA visible with other SS addition in garden soil. In addition, whereas $Ag_2S$ brought by SS caused only a transitory change in the diversity of the active microbial communities in agricultural soil, in garden soil, this change persisted until the end of experiment. Thus, the response of microbial communities to contamination by $Ag_2S$, derived from AgNPs, varied from one soil to another. The results of studies of the effects of $Ag_2S$ are therefore very difficult to generalize. $Ag_2S$ brought by SS can disturb at least in the medium term the active microbial communities in certain type of soil and thus affect certain activities such as DEA and therefore destabilize the nitrogen nutrient cycle. The spreading of SS containing Ag could sometimes disturb the proper functioning and fertility of soils.

**Keywords**

Silver nanoparticles, sewage sludge, microorganisms, diversity, NEA, DEA.

**Highlights**

- NEA is not affected by $Ag_2S$ (AgNPs-derived) via sewage sludge supply for both soils
- DEA decreased in one type of soil after sewage sludge containing $Ag_2S$ (AgNPs-derived)
- Active microorganisms' diversity is affected transiently by Ag in agricultural soil
- The impact of Ag is stronger on the active microbial communities of garden soil
- Overall, AgNPs affects differently the microorganisms communities of both soils

## 1. Introduction

In the last two decades, more and more manufactured products, including consumer products, have included silver nanoparticles (AgNPs) in their composition (Gottschalk et al., 2013; McGillicuddy et al., 2017; Vance et al., 2015). The world production of AgNPs is between 135 and 420 tons/years (INERIS, 2016). Among others properties, they are essentially incorporated for their excellent biocidal properties in medical and pharmaceutical products, but also a lot in cosmetics, textiles, food packaging and household products (Bone et al., 2012; INERIS, 2016; Maillard and Hartemann, 2013; Morones et al., 2005; Reidy et al., 2013). AgNPs are not the most-produced nanoparticles (NPs) in terms of quantity but the most widespread nanomaterial. In 2013, 435 of 1835 referenced nanomaterial containing products included AgNPs (Vance et al., 2015). The life cycle of these products causes considerable releases of silver (Ag) in environment and especially in wastewater due to the numerous rinses and washes of products and / or surface containing them (McGillicuddy et al., 2017). Thirty-seven to 46 % of produced AgNPs ends up in the sewer system (Adam et al., 2018). The efficiency of technologies in wastewater treatment plant (WWTP) allows to trap in biosolids the majority of Ag species (around 90 %) (Kaegi et al., 2011; Tiede et al., 2010). However, sewage sludge (SS) are often recycled by spreading on agricultural land in Europe (approximately 40 %) (Eurostat, 2020). The soil is therefore the main place of deposit of these Ag species (Massarsky et al., 2014) with an estimation of 140 tons of Ag that enters in agricultural soils annually from spreading of SS (Blaser et al., 2008). The predominant Ag species in sewage sludge is silver sulfide ($Ag_2S$) (Kaegi et al., 2013, 2011; Kent et al., 2014; Levard et al., 2012; Lombi et al., 2013; Ma et al., 2014; Pradas del Real et al., 2016). Because of anaerobic bacteria in sewers and WWTPs produce sulfides (Kaegi et al., 2013), AgNPs, and even silver ions, react with sulfides to give $Ag_2S$. Due to the quantity of Ag released and the amount of SS produced and spread each year, it became important to assess the impact of Ag

provided in soils, that is to say not in AgNPs form, already well studied (see review from Courtois et al., 2019), but in sulfided form. Especially since Ag is used for its excellent antimicrobial properties, we can fear a negative effect of it on soil microflora, which could significantly affect the soil functions. Decreases in the global abundance and activities of soil microorganisms such as the nitrification activity with a sensibility of ammonia-oxidizing bacteria were observed upon exposure to AgNPs (Hänsch and Emmerling, 2010; He et al., 2016). Changes in the diversity of microbial communities in response to direct exposure of AgNPs, even with low doses (few micrograms or milligrams per kg), have also been reported by several studies (Kumar et al., 2014; Liu et al., 2017; McGee et al., 2017; Samarajeewa et al., 2017; Sillen et al., 2015). However, the response of microorganisms to exposure of $Ag_2S$ brought via SS is still too little studied to have a clear vision of the impact of the spreading of SS contaminated by Ag on the microflora of terrestrial ecosystems (Asadishad et al., 2018; Courtois et al., 2019; Doolette et al., 2016; Durenkamp et al., 2016). However, understanding the results of the few rare studies on microorganisms is not necessarily obvious. There is no change in the microbial community with high concentrations of sulfided Ag (Doolette et al., 2016; Durenkamp et al., 2016), but despite with low concentrations (7-12 mg $kg^{-1}$ dry soil) changes in certain activities such as nitrification may appear (Kraas et al., 2017; Schlich et al., 2018, 2017). Thus, it is necessary to deepen the knowledge on the effects of AgNPs and their derivatives brought by sewage sludge in soils, on soil microflora.

The objective of this study was to evaluate the effect, in the short and medium term, of the Ag species brought by the SS in soils on soil microorganisms, more precisely on the diversity of active microorganisms and the nitrification and denitrification activities. For that, SS was contaminated with silver (either AgNPs or $AgNO_3$) before carrying out an anaerobic digestion to mimic treatment in a wastewater treatment plant. The SS was then mixed with two types of soil (one agricultural, one of garden). The microorganisms within these microcosms

were then analyzed at different time steps (over 35 days) in order to observe the effects of these species of Ag on the microflora over time.

## 2. Materials and methods

### *2.1. Soils*

Two soils from the Haut de France region (France) were collected a few days before the beginning of the experiment. The first one, an agricultural soil called "Agricultural soil", was a slightly calcareous (presence of chalk granules) brown soil developed on wind-blown silts on chalky substrate (DRAAF, 2013) (GPS coordinates: 50°59'94.87, 3°15'04.75). The second one, a soil from a private garden from Wambrechies (same region) called "Garden soil", was an alluvial clayey soil (DRAAF, 2013).

The 20 cm layer of these soils was collected and sieved at 5mm. The heavy metal contents of these soils are known (Supplementary data table 1). Silver concentrations were below the detection level for the agricultural soil and 0.31 ± 0.61 mg kg-1 for the garden soil.

### *2.2. Sewage sludge*

Sewage sludge (SS) was selected after a monitoring described in Courtois et al. (2020b*)*. Thus, the sewage sludge containing the lowest level of contamination and the preferable C:N ratio was selected. A previous study showed that the SS coming from the same WWTP was a good source of nutrients for earthworms, without inducing a 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, supporting an agricultural area which uses the activated sludge technology (flow: 1000, population equivalents: 20 000). The SS was characterized by metal contents: 11.53 ± 1.43, 0.95 ± 0.22, 1.10 ± 0.24, 140.62 ± 22.55, 145.43 ± 37.38, 186.78 ± 59.13, 19.11 ± 9.02, 32.21 ± 5.86, 2510.36 ± 615.99 mg $kg^{-1}$ (average based

on measurements between March and December 2016) for Ag, As, Cd, Cr, Cu, Mn, Ni, Pb, Zn respectively. Before agricultural reuse, anaerobic stabilization of sewage sludge has been carried out at laboratory scale.

### *2.3. Silver species*

Silver nanoparticles and dispersant solutions used for the exposition were provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology (Schmallenberg, Germany), as in Courtois et al. (2020b). The AgNPs correspond to the NM300K, a standard reference material of the European Commission Joint Research Centre. Briefly, these spherical NPs without coating correspond to a colloidal dispersion with a nominal silver content of 10.2 % by weight, dispersed in 4 % w/w % each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20). NPs have a nominal size of approximately 15 nm. Transmission electron microscopy (TEM) indicated a size of 17 ± 8 nm (Klein et al., 2011) and smaller nanoparticles of about 5 nm are also present (Mendes et al., 2015).

The dispersant solution, also from Fraunhofer Institute for Molecular Biology and Applied Ecology, was used for controls.

The silver nitrate used was in solid form initially and it was diluted with sterile distilled water.

The solutions of nanoparticles and silver ions used were diluted with milliQ water to obtain a concentration close to 2 mg $mL^{-1}$ both.

### *2.4. Experimental scheme*

#### *2.4.1. Anaerobic digestion of sewage sludge*

A batch anaerobic digestion of sewage sludge was performed in four continuous stirred-tank bioreactors. The bioreactors were glass vats filled with 6 liters of sewage sludge,

maintained in mesophilic conditions, at a temperature of 37 °C and constantly mixed (180 rpm) using mechanical stirrer. Details of the equipment as well as methods of analysis following indicators: pH, volatile fatty acids, volatile solids, total solids, ammonium nitrogen, are described previously (Grosser, 2017). In a first bioreactor, the SS was introduced without any additives (AD-control). In a second bioreactor 40 mg $L^{-1}$ of NM300K AgNPs (AD-AgNPs) was spiked. In a third bioreactor, only a corresponding quantity of dispersant (AD-dis) was added. In a fourth bioreactor 40 mg $L^{-1}$ of $AgNO_3$ (AD-$AgNO_3$) was added. The choosing Ag concentration in bioreactors is linked to the maximum concentration of Ag which does not disturb anaerobic fermentation (Yang et al., 2012) (Full justification in Sup. data 2). After 7 weeks of anaerobic digestion (AD), the process has stabilized, thus bioreactors were stopped and digestates were centrifuged at 12100 rcf for 15 minutes. No freezing took place in order to preserve the sludge microflora.

*2.4.2. Soil Microcosms Design*

For the 2 different soils (prefix A for Agricultural soil and prefix G for Garden soil), 4 treatments were applied:

- AD-control: soil supplied with a SS digested without any addition (A_AD-control and G_AD-control)
- AD-AgNPs: soil supplied with a SS digested with addition of AgNPs in dispersant (A_AD-AgNPs and G_AD-AgNPs)
- AD-dis: soil supplied with a SS digested with dispersant solution (A_AD-dis and G_AD-dis)
- AD-$AgNO_3$: soil supplied with a SS digested with $AgNO_3$ (A_AD-$AgNO_3$ and G_AD-$AgNO_3$)

A realistic quantity of SS, based on the tolerated spreading quantities, was brought to the microcosms in one time: 60 g of fresh SS per kg of soil. The details of the choice of sludge added to microcosms are given in Sup. Inf. 3. The amount of Ag remaining in the SS after the fermentation process was estimated to 0.233 mg of Ag per g of fresh SS. Thus, the estimated amount of silver was 14 mg $kg^{-1}$ of Ag (details of this estimation in Sup. data 3).

For each soil, mixtures of soil and SS were made in triplicates and 330g were distributed in 96 plastic boxes with perforated lids (diameter: 10 cm; high: 12 cm) with 330g (fresh matter). Six earthworms *Eisenia fetida* were introduced per microcosm to study effect of AgNPs presented in another study (Courtois et al., 2020b). A set of 4 replicate boxes for each treatment and each soil (32 microcosms) were sacrificed after 7, 21 and 35 days. At T0, since the mixture was the same for all the boxes, only one sample was analyzed in 4 replicates.

After soil homogenization of each sacrificed microcosm, 60 g of soil were stored at 4°C for activities measurements and 2 g were immediately frozen using liquid nitrogen and stored at -80°C for RNA diversity analyzes.

### *2.5. Analysis*

2.5.1. Nitrifying and denitrifying enzyme activities

Nitrifying enzyme activity (NEA) and denitrifying enzyme activity (DEA) assays are incubations in environmental conditions close to optimal for the expression of the activity. NEA was determined using the method described by Wertz et al. (2007) modified from Smorczewski and Schmidt (1991). Samples of fresh soil (3 g equivalent dry mass) were incubated with 30 ml of a solution of $NaNO_2$ (5 µg of $N\text{-}NO_2^-$ $g^{-1}$ dry soil) for 30 h with gentle shaking (150 rpm) at 28 °C. During incubation, 1.5 mL of the suspensions was sampled at 0, 7, 22 and 37 h and

centrifuged (5000 rpm for 2 min). The supernatants were then filtered (0.2 µm pore size) and analyzed for $NO_2^-$ and $NO_3^-$ concentration using ionic chromatography.

The method used for DEA measurements was as described by (Patra et al., 2005) modified from (Smith and Tiedje, 1979), using 10 g equivalent dry mass of soil per sample. Briefly, DEA was determined as the rate of production of $N_2O$ during short-term (8 h) incubation under anaerobic conditions using a gas chromatograph (Agilent P200, USA). $KNO_3$ (200 µg of $NO_3^-$-N per gram of dry soil), glucose (0.5 mg of C per gram of dry soil) and glutamic acid (0.5 mg of C per gram of dry soil) were added to the soil samples and the soil moisture was brought to 100% water holding capacity. $N_2O$ concentration in the flask atmosphere was measured at 2, 4, 6 and 8 h. A linear rate of $N_2O$ production was always observed. The NEA and DEA assays were performed at the AME platform of Lyon University.

2.5.2. Diversity of the active microbial community

RNA extractions were performed using the RNA PowerSoil Total RNA Isolation Kit (Qiagen) following manufacturer's instructions followed by a DNAse step using the Ambion® TURBO DNA-free™. RNA has been transformed into complementary DNA (cDNA) by reverse transcription using the RT – M-MLV kit (Invitrogen). Then, the V4–V5 hypervariable region of the 16S rRNA gene targeting Bacteria and Archaea was amplified using the primers 515F, 5' – GTG YCA GCM GCC GCG GTA – 3' and 928R, 5' – CCC CGY CAA TTC MTT TRA GT – 3' (Eurogentec, Belgium). The primers were designed with MiSeq specific adaptors; 5' – CTT TCC CTA CAC GAC GCT CTT CCG ATC T– 3' for the forward and 5' – GGA GTT CAG ACG TGT GCT CTT CCG ATC T – 3' for the reverse. The reaction mixture included 1.40 µl of each primer (20 µM each), 28 µl AmpliTaq Gold Master mix (AmpliTaq Gold; 360 Master Mix Applied Biosystems), 2 µl of the sample DNA template at a concentration of 5

ng.µL$^{-1}$ and water qsp 55 µl. The PCR cycle conditions included initial denaturation at 95 °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 extension step at 72 °C for 10 min after cycling was complete. The amplicons were sequenced using an Illumina MiSeq (300 bp paired-end reads) platform at Genotoul Inc. (www.genotoul.fr, Toulouse, France).

### 2.5.3. Bioinformatics analysis

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 10,184 sequences.

### 2.5.4. Metal content in mixtures and TOC, DOC, NO3- characterization

Mixture samples were collected at day zero and at each time point. These samples were lyophilized and ground with a mortar and a pestle. The mineralization consisted of digestion of 300 mg of sample in 7 mL of concentrated $HNO_3$, using a Berghof microwave digestion system (speed wave MWS-2-Microwave pressure digestion). The obtained solution was dosed by

inductively coupled plasma optical emission spectrometry (ICP-OES; Thermo apparatus). Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Nickel (Ni), Lead (Pb), Zinc (Zn) and Ag were quantified.

Total organic carbon from soil samples was measured by following the ISO 10694:1995 method. The extraction of disponible organic carbon was performed according to (Vance et al., 1987) with $K_2SO_4$ 0.03 M (soil-solution 1 : 4, agitation 30 min, 20 °C). The concentration of soluble organic carbon was measured with a C-analyser (1010, O.I. analytical, Globalspec, NY, USA). The soil mineral nitrogen ($NO_3^-$) was extracted with 1 M KCl (1:3 soil/solution ratio), centrifuged for 15 min at 5800 g and filtered on a Durieux No.3 paper disc. The mineral N in the soil extracts was analysed by continuous flow colorimetry (TRAACS, 2000, Irama, Milwaukie, USA). The concentrations of NO3- were determined as described by (Kamphake et al., 1967).

### *2.6. Statistic analysis*

The normality (via Shapiro tests) and homocedasticity (via bartlett tests) of the data were tested in order to choose the appropriate statistical tests.

Non parametric ANOVA (ANalysis Of VAriance) were used to compare the metal contents in microcosms, using R package (R Core Team, 2008).

One-way ANOVA and Tukey's tests were performed with the PAST software (Hammer et al., 2001) to determine if there were significant differences in the NEA, DEA results obtained from different treatments. The coefficient of determination ($R^2$) indicating the part of variance of NEA or DEA explained by soil parameters was calculated by fitting the data to a linear regression on Excel software. Correlation tests using Spearman method were carried out to analyze the significance of the correlations between the NEA or DEA and the soil parameters, using R software.

Diversity community matrices were analyzed using primer software (PRIMER-E Ltd, Plymouth, UK). Rank similarity matrices were computed and used to construct 'maps' highlighting the similarity of community genetic structures among soil samples by non-metric MultiDimensional Scaling (MDS). One-way ANOSIM (ANalysis Of SIMilarity) was performed with the Primer software to test, for each sampling date, the significance of the treatment effect on the community structure. A SIMPER analysis was performed with the 90 most abundant OTUs to assess the contribution of each OTU (%) to the dissimilarity between each two groups. Non parametric ANOVA were used, on the 90 most abundant OTUs grouped by genera, in order to check the significant differences of the abundance of each genera between the 2 soils or the 4 treatments (in each soil), using R software.

## 3. Results

### 3.1. Metal contents in soil microcosms

In both soils, for the 2 treatments including addition of Ag (A_AD-AgNPs, A_AD-$AgNO_3$, G_AD-AgNPs and G_AD-$AgNO_3$), at the end of soil incubations (35 days), the concentrations of Ag was between 8.600 and 9.980 mg $kg^{-1}$ (Table 1). These concentrations were slightly less than the expected concentration following the estimation calculations (14 mg $kg^{-1}$ expected). These 4 conditions with Ag were statistically similar (p-values between 0.381 and 1.000). Statistical tests showed a significant difference of Ag concentrations between the microcosms with Ag and AD-control (p-values were 0.000 and 0.014 between A_AD-control and A_AD-AgNPs or A_AD-$AgNO_3$ respectively; and p-values were 0.003 and 0.026 between G_AD-control and G_AD-AgNPs or G_AD-$AgNO_3$ respectively), where Ag was detectable but at a concentration lower than 2.606 mg $kg^{-1}$.

At the end of exposure, As, Cr, Cu, and Ni concentrations were not significantly different between all treatments (Sup. data table 5). Significant differences were observed for Pb and Zn concentrations, higher in the garden soil compared to the agricultural soil (Sup. data table 5).

*Table 1: Silver concentrations in soil microcosms supplied with sewage sludge (mg $kg^{-1}$ of dry matter) and control microcosms, at the end of incubation (35 days). Asteriks show the significant differences between treatments with silver (AD-AgNPs and AD-$AgNO_3$) and the control treatments (Control and AD-control).*

| Soil | Treatment | Ag |
|---|---|---|
| Agricultural | control | below detection level |
| Agricultural | AD-control | 1.043 ± 0.297 |
| | AD-AgNPs | *9.980 ± 0.370 |
| | AD-$AgNO_3$ | *9.145 ± 2.797 |
| Garden | control | below detection level |
| Garden | AD-control | 2.606 ± 0.759 |
| | AD-AgNPs | *9.845 ± 0.500 |
| | AD-$AgNO_3$ | *8.600 ± 0.635 |

3.2. Effects of sewage sludge (containing or not Ag) supply on the Nitrifying Enzyme Activity

No effect of the dispersant supply via SS on the NEA was observed, this treatment is presented in supplementary data 6 for readability.

Overall, the SS supply led to a significant increase in NEA of both soils in all treatments compared to the soil without any addition (Figure 1). This increase was transitory in the garden soil (until 7 or 21 days depending on the treatment) and variable between sampling times and

treatments in the agricultural soil. In both soils, NEA was not different between soils supplied with SS containing Ag (AgNPs or $AgNO_3$) compared to the control soil supplied with SS only.

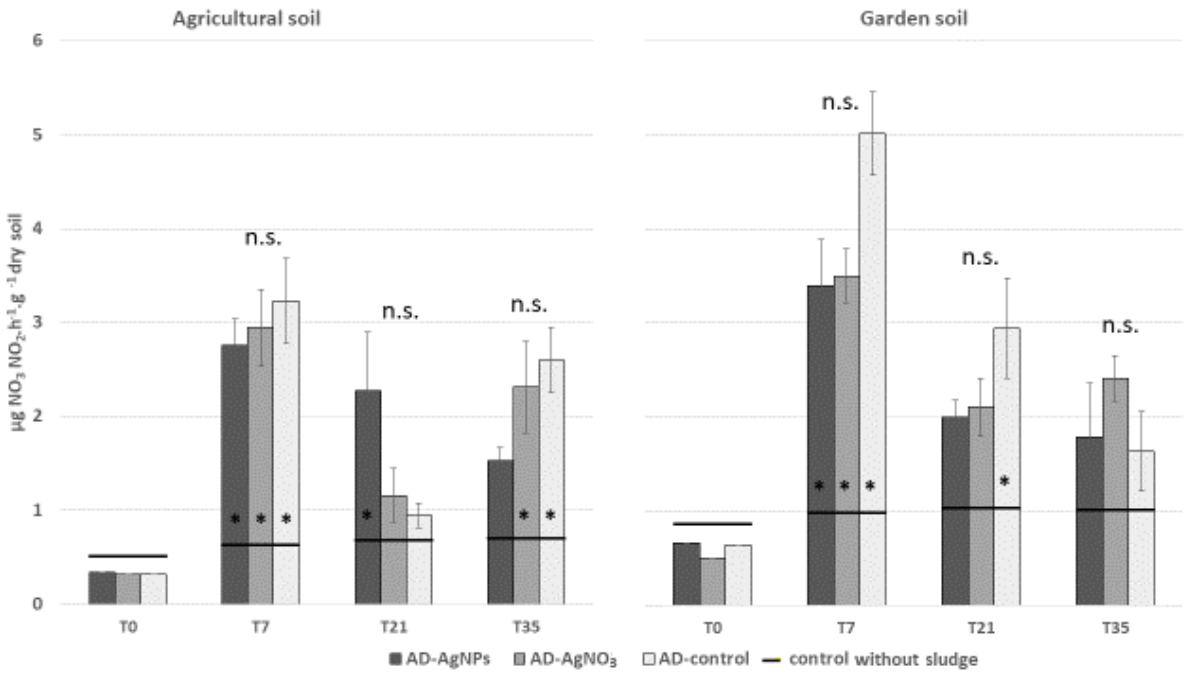


*Figure 1: Nitrifying Enzyme Activity in agricultural soil (left) and garden soil (right). The black lines correspond to the soil control without sewage sludge supply. T0, T7, T21 and T35 correspond to the sampling time in days. Asterisks show the significant difference between the treatment and the soil control without sewage sludge supply at $p < 0.05$. Different letters show the difference between the 3 treatments at each sampling time at $p < 0.05$. Error bars represent standard errors with n = 4. N.s. means no significant effect of the treatment.*

3.3. Effects of sewage sludge (containing or not Ag) supply on the Denitrifying Enzyme Activity

No effect of the dispersant supply via SS on the NEA was observed, this treatment is presented in supplementary data 7 for readability.

Overall, the addition of SS in both soils caused an increase of DEA, except in garden soil in AD-AgNPs treatment (Figure 2). More precisely, in the agricultural soil, SS increased

significantly the DEA until 7 days in AD-control, 21 days in AD-AgNPs and until the end of the experiment (35 days) for the AD-AgNO3 treatment, compared to the control without SS (Fig. 2, left). In the garden soil, SS increased significantly the DEA in AD-control and AD-$AgNO_3$ until the end of the experiment, compared to the control without SS (Fig. 2, right).

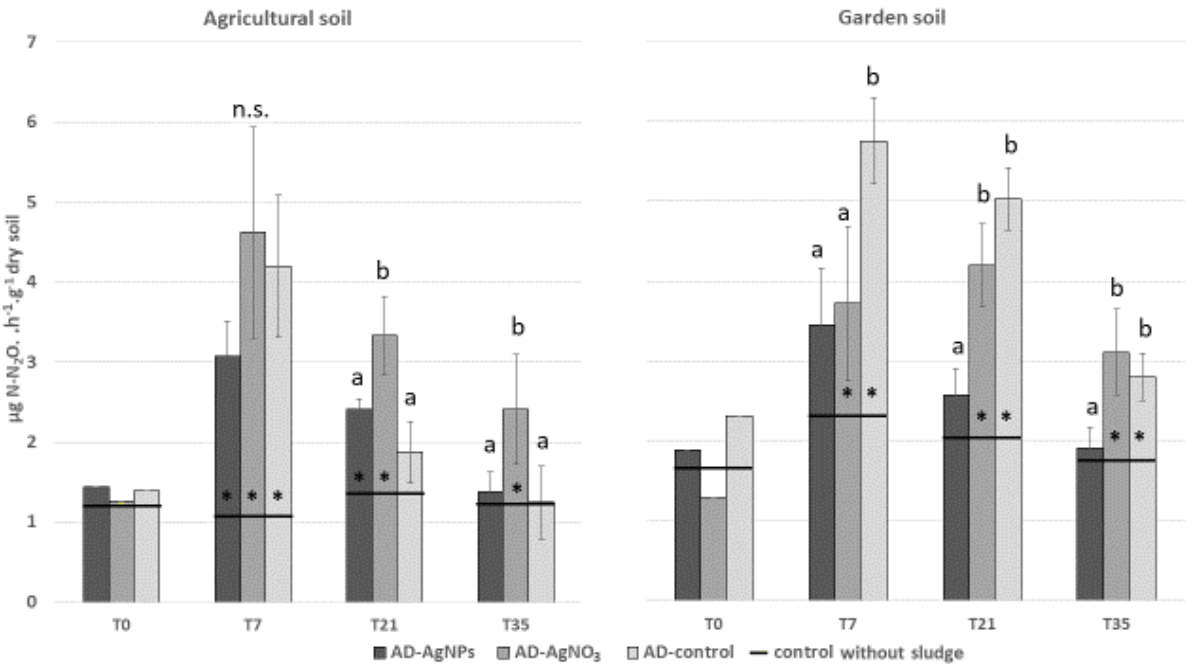


*Figure 2: Denitrification activity in agricultural soil (left) and garden soil (right). Control in black lines corresponds to the control treatment without sewage sludge. T0, T7, T21 and T35 mean the time point where DEA was measured i.e. at the initial time, after 7, 21 or 35 days of exposure. Asterisks showed the significant difference between the treatment and the soil control without addition of sludge at p < 0.05. Letters showed the difference between the 3 treatments at each time point at p < 0.05 with n=4.*

*Relationship between changes in DEA and soil environment parameters.*

At the end of experiment, in the agricultural soil, the variations of DEA were negatively correlated to the variations of the dissolved organic carbon (DOC) pool in soil ($R^2$ = 0.75; p-value = 0.000) (Table 2). In this soil, DEA was not significantly correlated to total organic carbon (TOC) or $NO_3^-$ pool.

At the end of experiment, in the garden soil, the variations of DEA were significantly and negatively correlated to the variations of the $NO_3^-$ pool ($R^2$ = 0.61; p-value = 0.016). In this soil, DEA was not correlated to DOC or TOC.

*Table 2: Correlations between potential denitrification activity and soil parameters (amount of $NO_3^-$, TOC, DOC). Only 3 treatments with sludge were included in these tests: AD-control, AD-AgNPs, AD-$AgNO_3$. The numerical value corresponds to the R2 associated with the linear relation between both compared parameters. It corresponds to the percentage of variations of DEA explained by the variations of the studied parameter. The sign + or - corresponds to the slope of the linear relationship, i.e. positive correlation (if +) or negative correlation (if -). Bold numbers with stars correspond to significant correlation according to the Pearson correlation test on R software. In this case, the p-value is indicated.*

| Microbial activity | Soil | $NO_3^-$ | TOC | DOC |
|---|---|---|---|---|
| DEA | Agricultural | 14.7 % - | 1.0 % + | **75.0 % - p-value < $2.2e^{-16}$** |
| | Garden | **61.3 % - p-value = 0.016** | 5.9 % - | 12.3 % - |

3.4. Effects of sewage sludge (containing or not Ag) supply on the diversity of the active microbial community

The agricultural and the garden soils had significantly different diversities of their active microbial community (Figure 3, p-value = 0.029). Besides, within each soil, the diversity of active microbial community was significantly different between each sampling dates (p-value = 0.029 for each comparison of sampling dates).

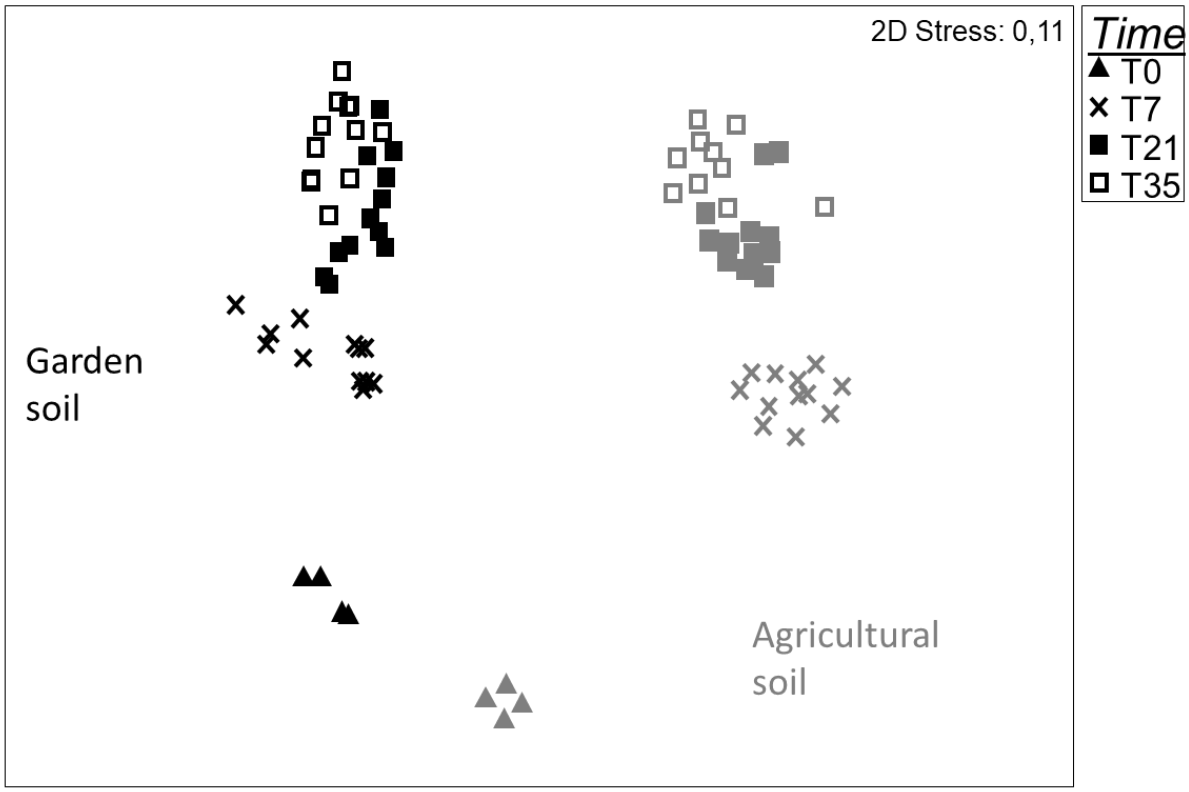


*Figure 3: nMDS plot illustrating distances in BrayCurtis similarity index between diversity of the active microbial communities of all treatments (except for AD-dis) and all sampling dates. T0 (triangle), T7 (cross), T21 (full square) and T35 (hollow square) corresponds to the sampling time in days. The agricultural soil is represented by gray symbols and the gargen soil is represented by black symbols.*

This difference of microbial communities between the agricultural and the garden soils was linked to a few genera of microorganisms (highlighted by non-parametric ANOVA). In the agricultural soil, there was more OTUs affiliated to genera *Comamonas, Azoarcus* and *Perlucidibaca.* In the garden soil, there was more OTUs affiliated to generas *Adhaeribacter, env. OPS 17 (Sphingobacteriales), Rhizobacter, Pseudoxanthomonas* and *Acidibacter* (see Sup. data 8).

In the agricultural soil, the diversity of the active microbial communities of the AD-$AgNO_3$ and AD-AgNPs treatments was significantly different from the one of the control treatment AD-Control during 7 and 21 days of incubation respectively (Figure 4 left and Table 3). At the end of the experiment after 35 days, the diversity of the active microbial communities was similar in all the treatments (i.e. no difference between AD-control, AD-$AgNO_3$ and AD-AgNPs). On the contrary, in the garden soil, the diversity of the active microbial communities of each treatment were all different to each other for each sampling time (i.e. significant differences between AD-control and AD-$AgNO_3$ and AD-AgNPs) (Figure 4 right and Table 3).

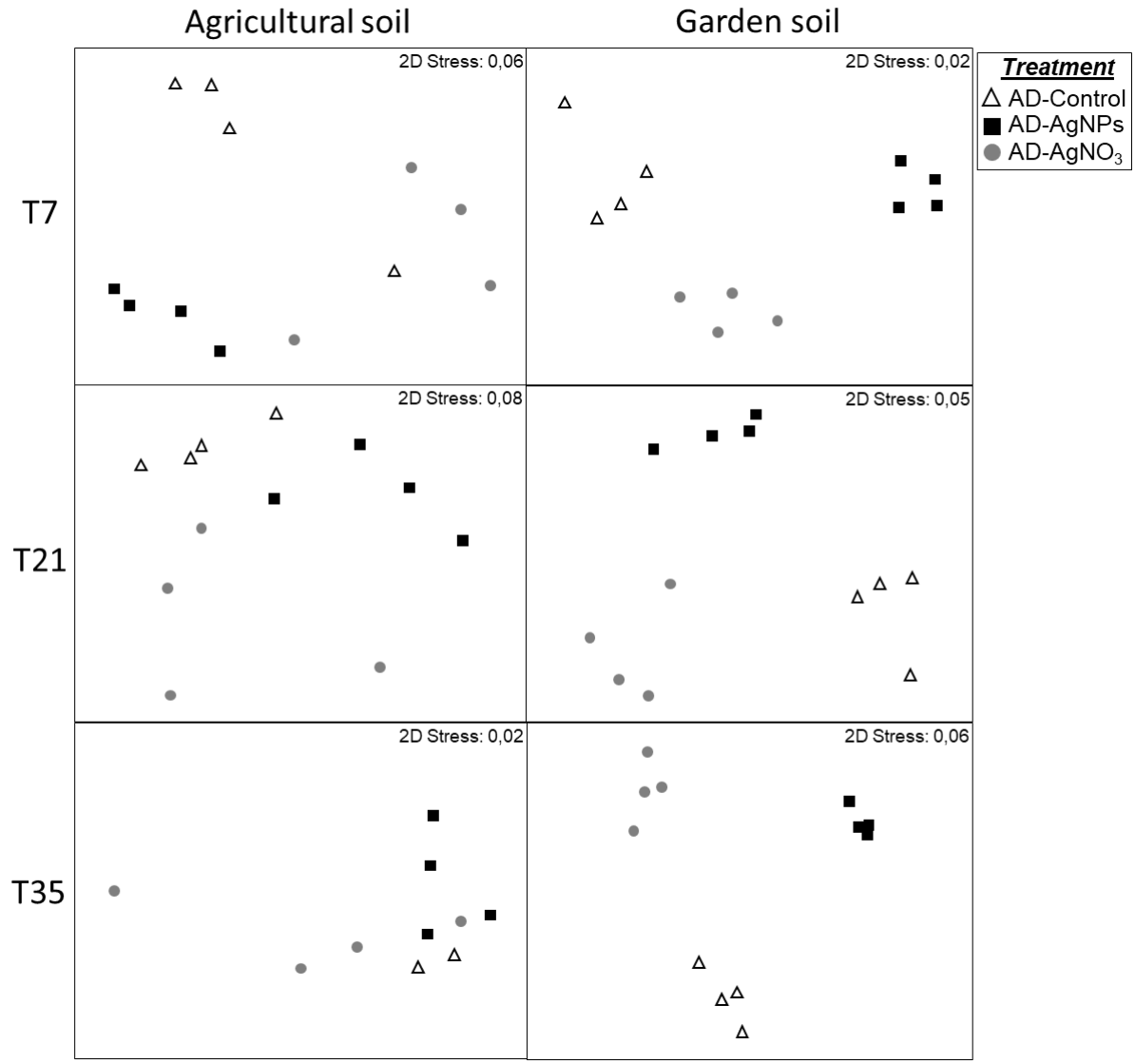


*Figure 4: nMDS plot illustrating distances in BrayCurtis similarity index between microbial communities of the 3 treatments (AD-control (triangle), AD-AgNPs (black square) and AD-$AgNO_3$ (gray circle)) in the 2 soils and at each sampling time (T7, T21 and T35 correspond to 7, 21 and 35 days of exposure).*

*Table 3: Results (p-values) of the one-way ANOSIM for comparisons of the diversity of the active microbial community between each pair of treatments for each sampling date (7, 21 and 35 days).*

| | Agricultural soil | | | Garden soil | | |
|---|---|---|---|---|---|---|
| | T7 | T21 | T35 | T7 | T21 | T35 |
| AD-AgNPs vs AD-control | 2.9 % | 2.9 % | n.s. | 2.9 % | 2.9 % | 2.9 % |
| AD-$AgNO_3$ vs AD-control | 2.9 % | n.s. | n.s. | 2.9 % | 2.9 % | 2.9 % |
| AD-AgNPs vs AD-$AgNO_3$ | n.s. | n.s. | n.s. | 2.9 % | 2.9 % | 2.9 % |

In both soils, the SIMPER results showed that OTUs contributing to the dissimilarity of couples of treatments were affiliated with environmental genera conventionally found in soils like *Arenimonas* (contribution between 1.26 and 6.06 %), *Flavobacterium* (contribution between 1.90 and 10.40 %), *Fluviicola* (contribution between 1.32 and 5.31 %), *Haliangium* (contribution between 1.47 and 3.09 %), *Lysobacter* (contribution between 1.26 and 6.99 %), *Nannocystis* (contribution between 1.40 and 2.37 %), *Perlucidibaca* (contribution between 1.28 and 7.99 %), *Permianibacter* (contribution between 1.49 and 3.44 %), *Pseudomonas* (contribution between 1.26 and 8.28 %), *Pseudoxanthomonas* (contribution between 2.03 and 3.12 %), *Ramlibacter* (contribution between 1.67 and 5.03 %), *Rhizobacter* (contribution between 1.33 and 3.19 %), *Sorangium* (contribution between 2.21 and 3.10 %) and *Thiobacillus* (contribution between 1.33 and 4.23 %); knowing that the maximum contribution of an OTU has not exceeded 6.84 %.

One genus and one class in connection with the fermentation of SS contributed also often to the difference between the treatments, in two soils: the *Methanosarcina* genus (contribution between 1.56 and 6.84 %) and the *Bacteroidetes vadinHA17* class (contribution between 1.45 and 5.83 %).

In agricultural soil, only at the start of exposure (T7), some genera of microorganisms showed a significant difference (observed with non-parametric ANOVA) in abundance between treatments. The *Nitrospira* genus had weaker abundance in AD-AgNPs (6 ± 3) compared to AD-control and AD-dis (9 ± 6) and the *Bacteroidetes vadinHA17* class had higher abundance in AD-AgNPs (103 ± 21) compared to all other treatments (8 ± 5) in agricultural soil at T7. At the end of exposure, in agreement with the nMDS results, there were no longer any significant differences in the abundance of OTUs between the treatments in agricultural soil.

In garden soil, some genera of microorganisms showed a significant difference (observed with non-parametric ANOVA) in abundance between treatments at the start of exposure, but were not detected over the weeks (Figure 5 and Sup. data 8). This is the case of the *Haliangium* genus in the garden soil that had weaker abundance in AD-AgNPs compared to AD-control and AD-dis at the start of experiment, as well as the *Thiobacillus* and *Methanosarcina* genera that had higher abundance in AD-AgNPs compared to 3 other treatments with SS at the start of experiment. The *Nitrosospira* genus had higher abundances in AD-$AgNO_3$ compared to 3 other treatments. In addition, some genera or classes showed significant differences in abundances between treatments during all the experiment. This was the case of the *Pseudomonas* genus that had higher abundances in AD-$AgNO_3$ compared to AD-control and AD-dis. The *Bacteroidetes vadinHA17* class had higher abundance in AD-AgNPs compared to all other treatments.

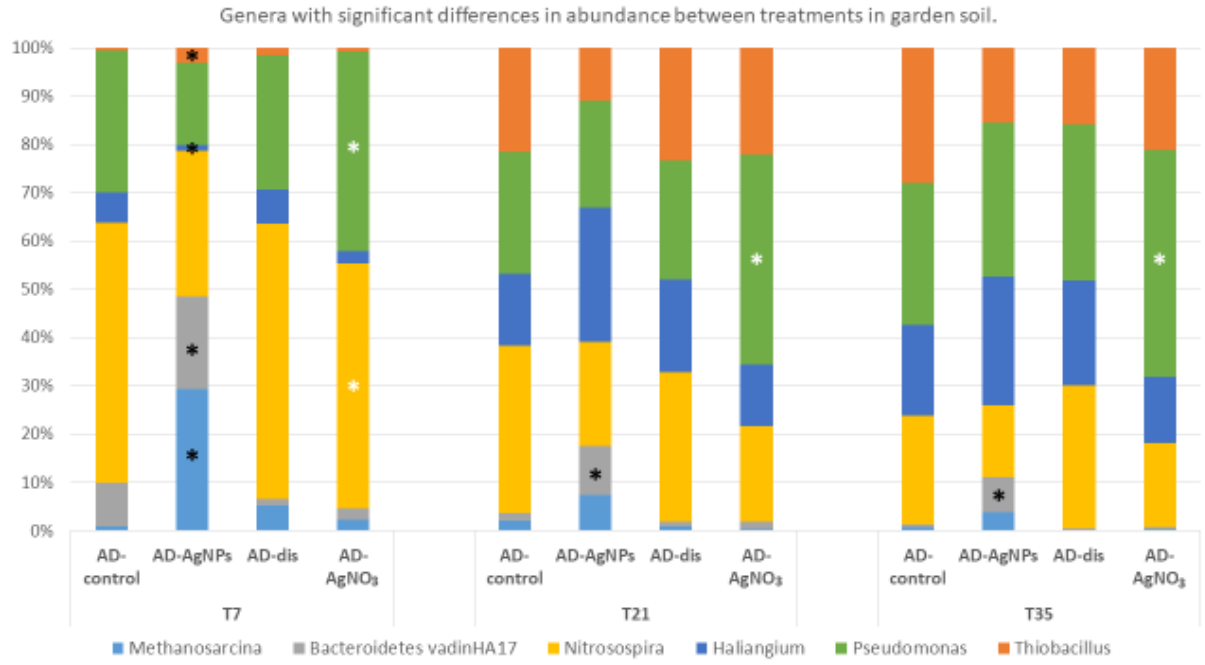


*Figure 5: Relative abundance of genera of microorganisms for which significant differences in abundance were visible with non-parametric ANOVAs. Black stars show the significant differences of abundance between G_AD-AgNPs and others treatments at a given time. White stars show the significant differences of abundance between G_AD-$AgNO_3$ and others treatments at a given time.*

## 4. Discussion and conclusion

Thus, the results of this study confirm that the addition of SS, and especially the supply of nitrogen, leads to a transient increase in NEA (Calleja-Cervantes et al., 2017), rather similar in the two soils even if it is more variable in agricultural soil compared to garden soil. The correlation between NEA and nitrogen pool was probably not significant because the nitrogen measurement corresponds to a pool and not a flow. In both soils, the 9-10 mg $kg^{-1}$ of Ag present in the SS did not influence the NEA response. Therefore, the NEA appears to be "resistant" to such concentration Ag sulfide. Nevertheless, this activity is generally considered to be rather

sensitive to disturbances and / or contaminants (like metals ; Kapoor et al. (2015)), due to the fact that it is carried out by a limited number of microorganisms, auto and mixotrophs. Moreover several studies with AgNPs have shown that ammonium oxidizing bacteria of agricultural pastureland soil were sensitive to AgNPs (McGee et al., 2017) and that the nitrification activity decreased with AgNPs contamination in natural soil (He et al., 2016). This phenomenon was also observed with $Ag_2S$; indeed, Kraas et al. (2017) showed a strong inhibitory effect of 8 mg $kg^{-1}$ of sulfided Ag (sulfidation during digestion of SS) on the potential ammonium oxidation activity in mesocosm with natural soil but which was visible from the $60^{th}$ or $90^{th}$ day of exposure (no measurement earlier). Strong inhibitions of AOB activity were also observed after applications of 7-12 mg $kg^{-1}$ of Ag sulfided (during a digestion) in SS mixed with a natural soil in mesocosms in Schlich et al. (2018) and Schlich et al. (2017), however, here also, these inhibitions were visible after a certain exposure time in the study of 2018: after 140 days of incubation while it was not significant after 60 and 90 days (no initial measurement); and in the study of 2017, the inhibition of AOB activity was significant on day 0 and day 60 (and thereafter) but not on day 30. Our measurement times are therefore not entirely comparable with these studies and the single point comparable is 30 days in Schlich et al. (2017) showing, as in the present study, the lack of effect of $Ag_2S$ on this activity. If the harmful effects of $Ag_2S$ on the microorganisms which carry out the nitrification appear gradually, for example, following the slow formation of $Ag^+$ ions (due to the stability of $Ag_2S$), then it is possible that the 5 weeks of exposure carried out in this present study were not sufficient to detect this effect.

The addition of SS, and especially the supply of organic matter and nitrogen, also leads to an increase in DEA, already observed previously (Calleja-Cervantes et al., 2017; Enwall et al., 2005), quite different in the two studied soils, transient in AD-control treatment in agricultural soil this time but lasting in AD-control treatment in garden soil. However, the

correlations between DEA and TOC, DOC and $NO_3$ pool were either insignificant or negative, which is contrary to our expectations (Burford and Bremner, 1975). This same result was observed by (Wu et al., 2020). They hypothesize that it is the NEA that determine the DEA since both activities are coupled. While in agricultural soil, the sulfide Ag from initially AgNPs in AD-AgNPs SS had no effect on DEA compared to AD-control treatment; in garden soil, it caused an inhibition of the rise of DEA normally observed after a SS application (visible in G_AD-control, G_AD-dis and also in G_AD-$AgNO_3$). It seems that the very large quantity of nitrogen in the garden soil played a role in this inhibition of DEA (negative significant correlation): it is possible that the strong nitrogen adding in garden soil has saturated the DEA. Thus, the addition of AgNPs before the anaerobic digestion of SS therefore has a medium-term impact (35 days after mixing of SS in the soil) on the DEA in certain soils: here the garden soil only. The denitrification is a less studied process in Ag contamination scenarii but it has been shown that Rhizobiales, which perform denitrification (O'Hara and Daniel, 1985), are sensitive to AgNPs (Kumar et al., 2014). A recent study has shown that the $Ag_2S$ provided by a sludge in natural soil, with a final concentration of 30 mg $kg^{-1}$ of Ag, lead to a decrease in denitrification in the soil in the presence of earthworms (Wu et al., 2020). Also, they observed that in the presence of epigeic earthworms (*E. fetida* like in our study), the relative abundance of denitrifying microbial genera was reduced both in the soil and in the earthworm intestines. With no earthworms in the soil, the denitrification results were quite different. Earthworms have been shown to influence $N_2O$ emissions (Lubbers et al., 2013), thus it was probable that although $Ag_2S$ is not very bioavailable to earthworms, its ingestion by the earthworms affects their intestinal microbiota and this would have repercussions on the soil microflora.

Indeed, the differences in the diversity of the active microbial communities from the start of the experiment showed that microbial diversity could be involved in this inhibition of DEA on garden soil. Indeed, the active microbial communities are very distinct between the two soils.

These communities are more or less sensitive and provide soils with variable characteristics and functionalities which mean that these two soil microbial communities may not respond in the same way to the same disturbances. Active microbial communities in agricultural soil showed greater resilience following disturbance as significant community differences between treatments diminished and disappeared after 5 weeks in agricultural soil. On the other hand, the communities of microorganisms active in the garden soil were less resilient to the disturbances undergone since at the end of the 5 weeks of incubation, there remained significant differences between the communities in the 4 treatments in the garden soil.

Indeed, the microbial communities between treatments in garden soil are distinguished by the abundance of certain genera like *Haliangium*, *Thiobacillus* and *Methanosarcina* (especially in the beginning of experiment), and the *Bacteroidetes vadinHA17* class (during all the experiment). The *Haliangium* genus are part of *Myxobacteria*, resistant gram-negative bacteria from soil able to resist to environmental stress factors like desiccation (Fudou et al., 2002). The *Methanosarcina*, anaerobic methanogen archaea, are current in digester of SS (Choi et al., 2018; Vitez et al., 2020), like the bacteria of *Bacteroidetes vadinHA17* class, which are fermenting bacteria degrading organics and fermenting carbohydrates (Walter et al., 2019; Wang et al., 2019). *Thiobacillus* genus corresponds to gram-negative bacteria, aerobic and sometimes anaerobic, from terrestrial or marine habitats, able to oxidize sulfur (Kadner, 1999; Rawlings and Kusano, 1994) and often associated to WWTPs since they participate to the metal leaching activity in SS (Blais et al., 1993).

Surprisingly, we have shown in a previous study that at the end of the SS fermentation in bioreactor and for 5 weeks after sludge-soil mixing, Ag was in sulfided form whether it had been supplied as AgNPs or $AgNO_3$ at the start of anaerobic digestion (Courtois et al., 2020b). Thus, at the start of fermentation, AgNPs being a very powerful biocide, thanks to both their nanoparticulate properties and their release of $Ag^+$ ions (Kędziora et al., 2018), may have reduce

the populations of the more sensitive bacteria present in SS, before being sulfided. This offered the possibility to resistant bacteria and / or fermentative, the possibility of multiplying strongly by occupying the ecological niches released. It is then probable that the development of these 3 genera / classes of bacteria took place, in part, to the detriment of groups of denitrifying bacteria. Thus, the SS enriched in AgNPs perhaps contained more resistant bacteria than the 3 other sludge. Thus, the effect of AgNPs at the beginning of fermentation process would impact on the microbial communities of the SS and this would further influence the active microbial communities after mixing in the soil for at least a few weeks. Indeed, Morones et al. (2005) also showed that 30 minutes were sufficient for the AgNPs to penetrate into microorganisms, in addition to the toxicity exerted by the release of $Ag^+$ ions (Courtois et al., 2019). It would be interesting to be able to study the microbial communities within SS that has undergone fermentation with and without AgNPs, and ideally at different stages of fermentation, in order to verify this hypothesis and assess the rapidity of toxicity of AgNPs.

*Conclusions*

This study shows that to understand the impact of releases of AgNPs into the environment, it is important to adopt an experimental protocol that is precise and as close as possible to what is happening in reality. Attention needs to be paid to the effects on organisms of AgNPs transformed during treatment of sewage sludge. The study of non-sulfided AgNPs has already shown that the results obtained were variable (Courtois et al., 2020b), hence the importance of carrying out a digestion of the SS before carrying out an ecotoxicological study. This work also shows that the study of silver ions, even if they have undergone transformations that offer them the same speciation as AgNPs at the end of fermentation, also leads to different results. Also, this study reminds us of the importance of long-term studies, lasting a few months

at least, since a few weeks do not allow to see whether the early effects disappear completely or to detect later effects.

Also, this study shows that AgNPs, released into the environment, transformed into Ag sulfides in wastewater treatment plants, and then landing in soils through the spreading of sewage sludge, even if they appear in a less toxic form when they land in the soil, can influence the microorganisms in real conditions. Ag sulfide can influence the microbial communities of the soil and sometimes also some activities (DEA) in the most "sensitive" soils. Surprisingly, it is not the most sensitive activities that are affected in the first place. A destabilized nitrogen cycle can lead to a disturbance in the availability of nitrogenous nutrients for plants. This could potentially destabilize the functioning of soils and possibly affect the fertility of cultivated soils. This impact of $Ag_2S$ from AgNPs would be due to the very early impact of AgNPs on the microbial communities of the SS which took place rapidly at the start of fermentation. AgNPs therefore have an impressive reactivity towards microorganisms which can leave traces in soils, at least in the medium term.

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**Supplementary data**

*Sup. data table 1: Metal contents of the agricultural and the garden soils (average based on triplicate samples taken in April 2018).*

| Metal concentrations (mg $kg^{-1}$ dry matter) | Ag | As | Ba | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|
| Agricultural soil | 0.00 ± 0.00 | 5.09 ± 0.08 | 34.53 ± 0.87 | 0.25 ± 0.02 | 37.98 ± 1.88 | 20.80 ± 0.22 | 14.40 ± 0.18 | 51.03 ± 1.78 | 60.28 ± 1.01 |
| Garden soil | 0.31 ± 0.61 | 5.68 ± 0.41 | 52.18 ± 5.20 | 0.33 ± 0.09 | 39.63 ± 4.98 | 43.13 ± 1.24 | 13.30 ± 0.36 | 99.55 ± 20.02 | 173.30 ± 12.85 |

*Sup. data 2: Justification for choosing silver concentration in bioreactors*

Doses of sludge introduced in microcosms was choosen according to the legislation. French legislation is more strict than European legislation (Directive 86/278/ECC, 1986), it lays down maximum quantities to be spread over a period of time. It is thus possible to apply up to 30 tons of dry matter per hectare over a period of 10 years (Circular DE / GE n ° 357 of 03/16/99, 1999). The soil density being 1.25 g/cm3 and the average depth of incorporation of sludge into agricultural soil is about 8 cm (typically 5 to 15cm), the soil mass that is mixed with sludge over one hectare then corresponds to about 1000 tons (density = mass/volume). Thus, on a one-year perspective only, it is possible to apply 3t of MS sludge per 1000 t of soil i.e. 3g of MS sludge per kilo of soil. Sludge contains about 85% water, so the wet sludge mass allowed for spreading is close to 20 g of fresh sludge per kg of soil per year. However, European legislation allows the addition, at one time, of significantly higher sludge quantities on agricultural soil, since it is possible to place up to 30t of MS sludge per hectare per decade. It is then legally possible to apply the equivalent of several years in one time. For this experiment, it was decided to bring in once the quantities of sludge that can be administered over 3 years. Thus, 60g of

fresh sewage sludge per kg of fresh soil has been added. The microcosms were then filled with 330g of this mixture.

*Sup. data 3: Justification for choosing sewage sludge doses in microcosms*

Dose of sludge introduced in microcosms was chosen according to the European legislation stating that it is possible to apply up to 30 tons of dry matter per hectare for a period of 10 years (Circular DE / GE n ° 357 of 03/16/99, 1999). The density of soil used in this study was 1.25 g.cm3 and the average depth of incorporation of SS into agricultural soil is about 8 cm (typically 5 to 15 cm). Thus, the soil mass that is mixed with SS over one hectare corresponds to about 1000 tons (density = mass/volume). Therefore, on a one-year perspective only, it is possible to apply 3 tons of sludge (dry matter) per 1000 tons of soil i.e. 3 g of MS sludge per kg of soil. Assuming that dewatered SS still contains around 85% water, the mass allowed for spreading is close to 20 g of fresh SS per kg of soil per year. However, European legislation allows the addition, at one time, of significantly higher SS quantities on agricultural soil, since it is possible to place up to 30 tons of MS sludge per hectare per decade. It is then legally possible to apply the equivalent of several years in one time, i.e. it is possible to apply 200 g of fresh SS per kg of soil in one time, for a decade.

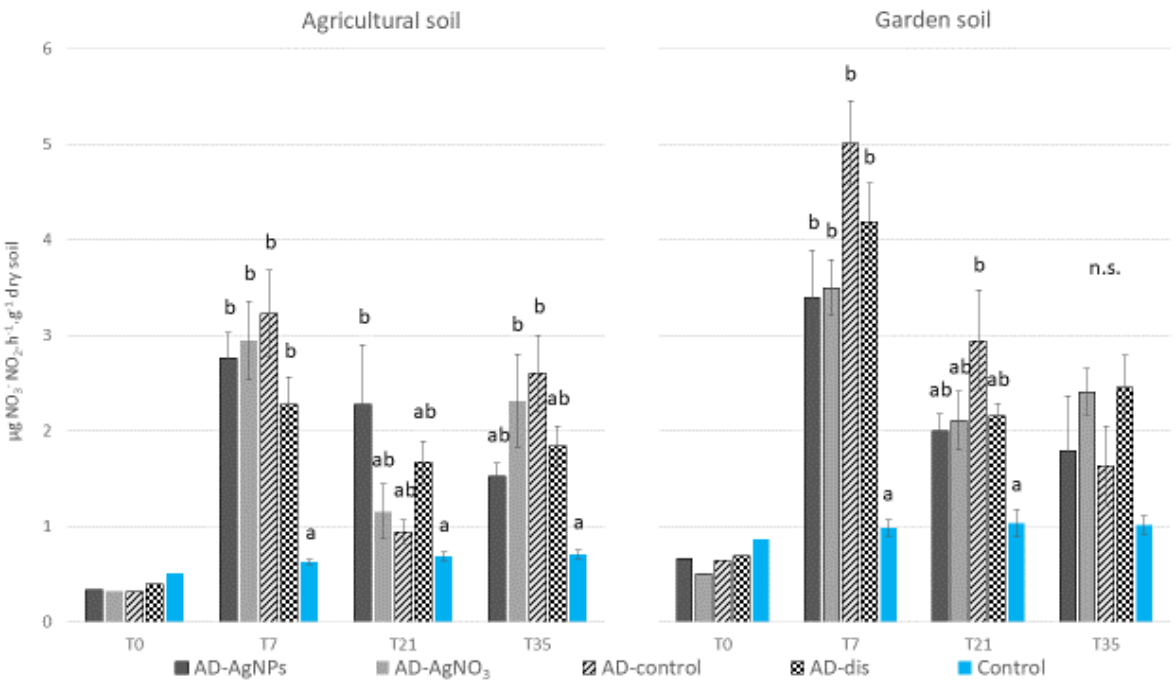
*Sup. data 4: Justification for estimating of Ag concentration in sewage sludge.*

Kaegi et al. (2011) and Tiede et al. (2010) showed that the sludge treatment allows trapping around 90% of Ag in biosolids. In our situation, approximately 216 mg of Ag (from the 240 mg initially added) should have stayed in SS. After one month of fermentation, there was around

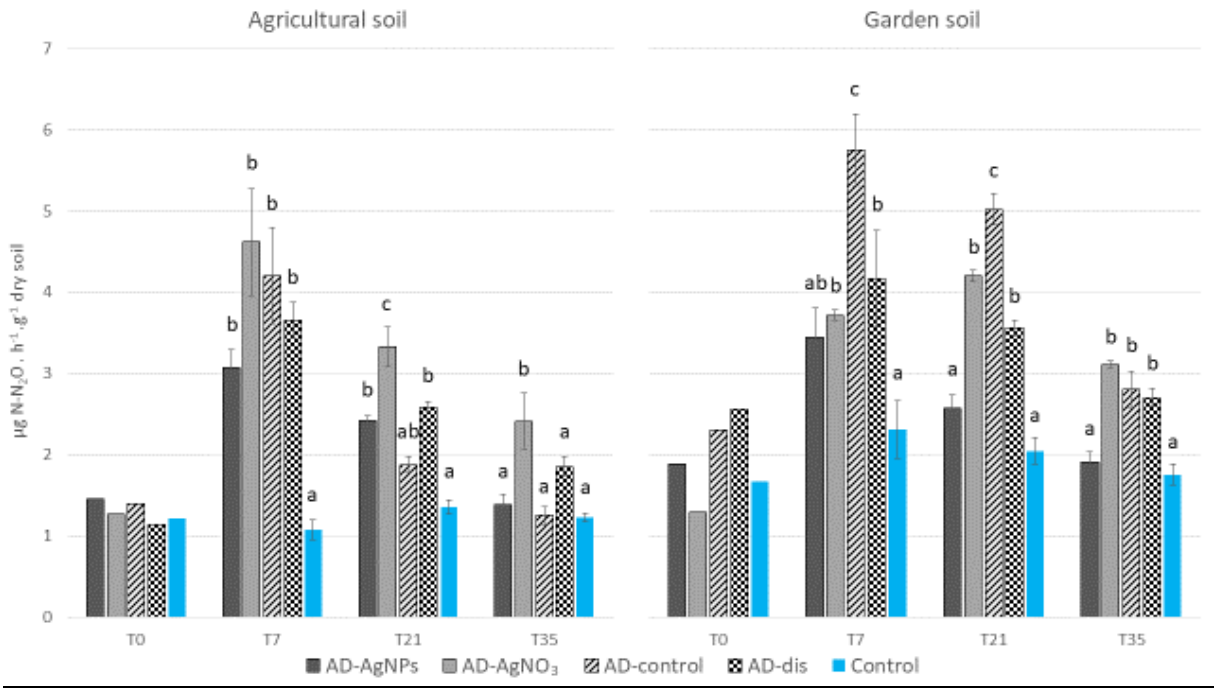
910 g of fresh SS in each bioreactor. Consequently, the addition of 60, 120 and 200 g of fresh SS per kilogram of fresh soil in microcosms corresponded to the addition of 14, 28 and 47 mg $kg^{-1}$ of Ag depending to the time perspective.

*Sup. data table 5: Metals concentrations in soil microcosms supplied with sewage sludge (AD-AgNPs and AD-$AgNO_3$) and control microcosms (AD-control and AD-dis), at the end of incubation (35 days), in mg $kg^{-1}$ of dry matter. Asteriks show the significant differences between treatments with silver (AD-AgNPs and AD-$AgNO_3$) and the control treatments (Control and AD-control).*

| Soil | Treatment | **Ag** | As | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|
| Agricultural | control | **0.000 ± 0.000** | 5.083 ± 0.029 | 0.277 ± 0.161 | 38.750 ± 3.543 | 20.175 ± 0.727 | 13.600 ± 0.469 | 49.100 ± 0.985 | 60.250 ± 0.635 |
| | AD-control | **1.043 ± 0.297** | 4.839 ± 0.221 | 0.235 ± 0.045 | 38.600 ± 0.794 | 21.733 ± 0.289 | 13.700 ± 0.173 | 49.267 ± 1.401 | 78.867 ± 0.208 |
| | AD-dis | | | | | | | | |
| | AD-AgNPs | ***9.980 ± 0.370** | 4.674 ± 0.168 | 0.186 ± 0.010 | 37.325 ± 2.210 | 21.375 ± 0.960 | 13.300 ± 0.258 | 47.725 ± 1.520 | 80.000 ± 0.392 |
| | AD-$AgNO_3$ | ***9.145 ± 2.797** | 4.679 ± 0.083 | 0.100 ± 0.067 | 37.700 ± 1.203 | 21.025 ± 0.350 | 12.700 ± 0.258 | 49.025 ± 3.809 | 75.450 ± 1.964 |
| Garden | control | **0.000 ± 0.000** | 6.305 ± 1.020 | 0.384 ± 0.072 | 35.350 ± 2.144 | 43.450 ± 0.370 | 15.250 ± 1.529 | 90.000 ± 6.393 | 166.900 ± 11.876 |
| | AD-control | **2.606 ± 0.759** | 5.128 ± 0.263 | 0.301 ± 0.017 | 35.575 ± 2.139 | 43.9000 ± 0.700 | 13.950 ± 0.915 | 83.900 ± 3.251 | 176.567 ± 6.408 |
| | AD-dis | | | | | | | | |
| | AD-AgNPs | ***9.845 ± 0.500** | 5.088 ± 0.164 | 0.250 ± 0.027 | 37.550 ± 5.114 | 37.425 ± 0.403 | 12.975 ± 0.556 | 79.400 ± 5.285 | 167.550 ± 4.138 |
| | AD-$AgNO_3$ | ***8.600 ± 0.635** | 4.988 ± 0.672 | 0.110 ± 0.133 | 37.775 ± 2.830 | 34.300 ± 0.572 | 12.050 ± 0.794 | 78.933 ± 9.416 | 143.075 ± 14.275 |




*Sup. data figure 6: Nitrifying Enzyme Activity in agricultural soil (left) and garden soil (right). T0, T7, T21 and T35 correspond to the sampling time in days. Different letters show the difference between the 5 treatments at each sampling time at $p < 0.05$. Error bars represent standard errors with $n = 4$. N.s. means no significant effect of the treatment.*




*Sup. data figure 7: Denitrifying Enzyme Activity in agricultural soil (left) and garden soil (right). T0, T7, T21 and T35 correspond to the sampling time in days. Different letters show the difference between the 5 treatments at each sampling time at $p < 0.05$. Error bars represent standard errors with $n = 4$.*

| Time | T0 | | | | | | | | T1 | | | | | | | | T2 | | | | | | | | T3 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Soil | Agricultural | | | | Garden | | | | Agricultural | | | | Garden | | | | Agricultural | | | | Garden | | | | Agricultural | | | | Garden | | | |
| Treatment | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 | AD-control | AD-AgNPs | AD-dis | AD-AgNO3 |
| Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;Methanosarcina | 33 | 306 | 32 | 40 | 17 | 198 | 38 | 68 | 4 | 27 | 9 | 7 | 2 | 104 | 9 | 7 | 5 | 22 | 4 | 5 | 5 | 26 | 3 | 1 | 38 | 29 | 3 | 2 | 1 | 11 | 1 | 1 |
| Archaea;Thaumarchaeota;Soil Crenarchaeotic Group(SCG);unknown order;unknown family;unknown genus | 14 | 13 | 14 | 22 | 7 | 14 | 8 | 6 | 10 | 5 | 13 | 5 | 4 | 11 | 7 | 4 | 28 | 21 | 21 | 27 | 14 | 20 | 11 | 10 | 56 | 40 | 49 | 27 | 10 | 16 | 7 | 15 |
| Bacteria;Acidobacteria;Blastocatellia;Blastocatellales;Blastocatellaceae (Subgroup 4);RB41 | 50 | 19 | 55 | 38 | 28 | 20 | 32 | 29 | 23 | 25 | 21 | 16 | 39 | 31 | 51 | 33 | 29 | 23 | 30 | 27 | 33 | 33 | 36 | 30 | 28 | 24 | 44 | 34 | 36 | 51 | 46 | 36 |
| Bacteria;Acidobacteria;Subgroup 6;unknown order;unknown family;unknown genus | 26 | 25 | 40 | 27 | 32 | 10 | 18 | 14 | 6 | 8 | 8 | 7 | 11 | 5 | 16 | 7 | 7 | 4 | 6 | 7 | 14 | 7 | 10 | 6 | 5 | 5 | 10 | 5 | 15 | 7 | 11 | 5 |
| Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Micrococcaceae;Multi-affiliation | 80 | 70 | 56 | 46 | 65 | 51 | 96 | 54 | 31 | 12 | 38 | 23 | 6 | 29 | 50 | 21 | 30 | 12 | 25 | 20 | 36 | 8 | 12 | 13 | 30 | 32 | 29 | 15 | 15 | 5 | 12 | 7 |
| Bacteria;Actinobacteria;Actinobacteria;Propionibacteriales;Nocardioidaceae;Multi-affiliation | 24 | 31 | 31 | 31 | 21 | 17 | 36 | 24 | 10 | 6 | 21 | 10 | 2 | 19 | 15 | 6 | 13 | 10 | 11 | 11 | 15 | 7 | 5 | 4 | 23 | 22 | 16 | 7 | 8 | 4 | 4 | 1 |
| Bacteria;Actinobacteria;Thermoleophilia;Gaiellales;Gaiellaceae;Gaiella | 4 | 4 | 11 | 7 | 27 | 37 | 58 | 39 | 3 | 1 | 4 | 1 | 4 | 29 | 30 | 13 | 3 | 2 | 2 | 3 | 35 | 21 | 15 | 13 | 8 | 6 | 8 | 4 | 28 | 16 | 19 | 11 |
| Bacteria;Actinobacteria;Thermoleophilia;Solirubrobacterales;Solirubrobacteraceae;Solirubrobacter | 17 | 16 | 24 | 17 | 28 | 22 | 35 | 26 | 7 | 3 | 11 | 6 | 3 | 19 | 19 | 8 | 5 | 5 | 8 | 7 | 21 | 12 | 9 | 5 | 12 | 13 | 7 | 9 | 9 | 5 | 7 | 3 |
| Bacteria;Bacteroidetes;Bacteroidetes vadinHA17;unknown order;unknown family;unknown genus | 87 | 266 | 8 | 82 | 71 | 228 | 4 | 52 | 13 | 103 | 5 | 6 | 18 | 68 | 3 | 7 | 12 | 48 | 3 | 4 | 3 | 36 | 2 | 4 | 16 | 15 | 2 | 4 | 1 | 20 | 1 | 1 |
| Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Petrimonas | 53 | 41 | 9 | 33 | 25 | 35 | 6 | 15 | 21 | 24 | 5 | 14 | 22 | 24 | 1 | 26 | 15 | 11 | 4 | 4 | 7 | 3 | 1 | 4 | 2 | 4 | 1 | 5 | 2 | 2 | 0 | 2 |
| Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cyclobacteriaceae;Algoriphagus | 2 | 3 | 1 | 1 | 5 | 3 | 4 | 0 | 6 | 8 | 4 | 3 | 26 | 8 | 12 | 10 | 15 | 30 | 30 | 36 | 27 | 33 | 78 | 72 | 24 | 21 | 36 | 25 | 86 | 78 | 95 | 77 |
| Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Adhaeribacter | 25 | 18 | 38 | 29 | 82 | 56 | 78 | 54 | 34 | 38 | 33 | 20 | 71 | 85 | 95 | 60 | 38 | 45 | 39 | 30 | 56 | 94 | 116 | 89 | 45 | 55 | 57 | 30 | 129 | 168 | 199 | 98 |
| Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;unknown genus | 19 | 13 | 7 | 21 | 17 | 20 | 22 | 16 | 13 | 14 | 13 | 15 | 14 | 16 | 17 | 13 | 52 | 25 | 36 | 54 | 25 | 24 | 38 | 32 | 37 | 35 | 42 | 121 | 37 | 27 | 48 | 38 |
| Bacteria;Bacteroidetes;Flavobacteriia;Flavobacteriales;Cryomorphaceae;Fluviicola | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 3 | 3 | 0 | 1 | 1 | 0 | 7 | 63 | 26 | 35 | 3 | 2 | 18 | 1 | 111 | 44 | 40 | 37 | 6 | 6 | 11 | 34 |
| Bacteria;Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Flavobacterium | 24 | 23 | 26 | 10 | 42 | 37 | 50 | 27 | 129 | 209 | 190 | 217 | 103 | 83 | 63 | 122 | 153 | 361 | 299 | 297 | 123 | 219 | 198 | 342 | 378 | 398 | 398 | 223 | 333 | 431 | 322 | 362 |
| Bacteria;Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Chitinophagaceae;Filimonas | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 9 | 5 | 7 | 4 | 11 | 15 | 12 | 11 | 23 | 31 | 44 | 38 | 37 | 34 | 29 | 30 | 64 | 74 | 53 | 72 |
| Bacteria;Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Chitinophagaceae;unknown genus | 25 | 19 | 25 | 26 | 27 | 14 | 18 | 19 | 5 | 8 | 7 | 7 | 13 | 11 | 14 | 9 | 7 | 7 | 5 | 4 | 12 | 8 | 14 | 7 | 6 | 6 | 8 | 6 | 16 | 9 | 13 | 10 |
| Bacteria;Bacteroidetes;Sphingobacteriia;Sphingobacteriales;env.OPS 17;unknown genus | 1 | 0 | 1 | 0 | 4 | 2 | 5 | 4 | 1 | 2 | 2 | 1 | 49 | 15 | 32 | 17 | 5 | 9 | 7 | 8 | 26 | 63 | 43 | 63 | 8 | 10 | 9 | 11 | 56 | 65 | 46 | 55 |
| Bacteria;Firmicutes;Clostridia;D8A-2;unknown family;unknown genus | 162 | 78 | 127 | 178 | 116 | 68 | 38 | 84 | 29 | 42 | 28 | 31 | 28 | 28 | 13 | 33 | 29 | 22 | 21 | 17 | 4 | 12 | 2 | 7 | 8 | 7 | 4 | 8 | 2 | 4 | 2 | 3 |
| Bacteria;Multi-affiliation;Multi-affiliation;Multi-affiliation;Multi-affiliation;Multi-affiliation | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 77 | 40 | 34 | 96 | 1 | 1 | 4 | 2 | 48 | 14 | 18 | 48 | 0 | 4 | 1 | 3 | 23 | 18 | 11 | 26 |
| Bacteria;Nitrospirae;Nitrospira;Nitrospirales;0319-6A21;unknown genus | 113 | 105 | 118 | 101 | 127 | 114 | 129 | 137 | 22 | 22 | 20 | 20 | 42 | 58 | 56 | 52 | 26 | 18 | 22 | 25 | 69 | 71 | 60 | 66 | 21 | 28 | 37 | 27 | 57 | 64 | 67 | 73 |
| Bacteria;Nitrospirae;Nitrospira;Nitrospirales;Nitrospiraceae;Nitrospira | 2 | 1 | 2 | 1 | 7 | 2 | 0 | 4 | 15 | 6 | 17 | 8 | 2 | 6 | 6 | 4 | 24 | 23 | 31 | 26 | 4 | 4 | 2 | 1 | 29 | 30 | 40 | 17 | 2 | 2 | 2 | 2 |
| Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;Phenylobacterium | 5 | 1 | 6 | 0 | 9 | 4 | 9 | 9 | 13 | 12 | 9 | 10 | 18 | 14 | 13 | 20 | 12 | 10 | 10 | 14 | 13 | 12 | 15 | 11 | 11 | 10 | 10 | 17 | 13 | 8 | 10 | 12 |
| Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae;Multi-affiliation | 8 | 14 | 13 | 17 | 9 | 17 | 26 | 21 | 13 | 9 | 12 | 9 | 14 | 33 | 33 | 22 | 9 | 11 | 13 | 11 | 29 | 28 | 19 | 11 | 17 | 12 | 11 | 4 | 16 | 9 | 8 | 5 |
| Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Amaricoccus | 10 | 17 | 23 | 16 | 16 | 30 | 29 | 40 | 10 | 3 | 15 | 7 | 2 | 17 | 21 | 8 | 15 | 10 | 9 | 8 | 28 | 10 | 6 | 9 | 21 | 21 | 15 | 4 | 10 | 8 | 10 | 4 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Comamonas | 11 | 18 | 20 | 17 | 3 | 2 | 1 | 4 | 52 | 65 | 49 | 45 | 2 | 7 | 6 | 7 | 43 | 37 | 35 | 34 | 2 | 7 | 3 | 3 | 28 | 26 | 29 | 29 | 4 | 4 | 5 | 5 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Leptothrix | 5 | 3 | 8 | 4 | 14 | 5 | 6 | 11 | 9 | 9 | 6 | 6 | 11 | 11 | 11 | 9 | 12 | 17 | 15 | 9 | 15 | 14 | 19 | 15 | 13 | 10 | 15 | 8 | 16 | 7 | 16 | 12 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Multi-affiliation | 217 | 191 | 197 | 153 | 375 | 319 | 436 | 285 | 431 | 491 | 474 | 467 | 561 | 430 | 495 | 437 | 539 | 523 | 492 | 450 | 518 | 455 | 458 | 407 | 344 | 362 | 344 | 366 | 381 | 349 | 372 | 335 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Ramlibacter | 1 | 4 | 5 | 1 | 0 | 3 | 1 | 3 | 5 | 6 | 6 | 6 | 70 | 8 | 13 | 77 | 3 | 5 | 4 | 5 | 44 | 3 | 6 | 16 | 3 | 4 | 5 | 9 | 16 | 3 | 5 | 12 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Rhizobacter | 0 | 0 | 2 | 0 | 2 | 8 | 6 | 7 | 2 | 2 | 1 | 1 | 27 | 22 | 18 | 43 | 1 | 1 | 1 | 1 | 76 | 58 | 48 | 132 | 4 | 3 | 3 | 3 | 107 | 75 | 57 | 158 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;unknown genus | 61 | 55 | 58 | 52 | 132 | 114 | 120 | 126 | 94 | 144 | 87 | 68 | 164 | 155 | 167 | 165 | 87 | 107 | 92 | 68 | 127 | 163 | 154 | 135 | 76 | 91 | 88 | 51 | 94 | 109 | 118 | 124 |
| Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Multi-affiliation | 12 | 12 | 23 | 7 | 2 | 6 | 11 | 12 | 69 | 69 | 168 | 83 | 58 | 45 | 40 | 57 | 44 | 32 | 57 | 58 | 37 | 35 | 26 | 20 | 28 | 40 | 29 | 43 | 10 | 11 | 14 | 13 |
| Bacteria;Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;Thiobacillus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 18 | 19 | 36 | 61 | 1 | 11 | 3 | 2 | 108 | 89 | 124 | 158 | 44 | 38 | 56 | 62 | 63 | 74 | 46 | 167 | 47 | 43 | 28 | 56 |
| Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;Multi-affiliation | 0 | 2 | 2 | 6 | 6 | 2 | 7 | 3 | 24 | 15 | 15 | 28 | 13 | 6 | 17 | 11 | 16 | 10 | 13 | 9 | 12 | 7 | 15 | 4 | 8 | 9 | 9 | 9 | 12 | 5 | 14 | 6 |
| Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;Nitrosospira | 11 | 22 | 10 | 28 | 21 | 34 | 33 | 26 | 172 | 126 | 130 | 144 | 104 | 106 | 100 | 153 | 92 | 84 | 83 | 66 | 71 | 75 | 74 | 56 | 67 | 78 | 74 | 52 | 38 | 41 | 53 | 46 |
| Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Azoarcus | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 34 | 24 | 59 | 61 | 0 | 4 | 1 | 1 | 49 | 29 | 54 | 69 | 1 | 3 | 1 | 3 | 36 | 35 | 38 | 76 | 1 | 5 | 0 | 3 |
| Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Multi-affiliation | 9 | 4 | 2 | 4 | 1 | 5 | 1 | 2 | 12 | 14 | 37 | 41 | 13 | 15 | 5 | 14 | 29 | 46 | 44 | 71 | 21 | 29 | 7 | 12 | 36 | 35 | 24 | 89 | 19 | 44 | 3 | 14 |
| Bacteria;Proteobacteria;Betaproteobacteria;TRA3-20;unknown family;unknown genus | 6 | 1 | 7 | 7 | 2 | 3 | 7 | 2 | 7 | 4 | 2 | 4 | 4 | 2 | 6 | 3 | 19 | 14 | 15 | 13 | 8 | 9 | 11 | 8 | 24 | 21 | 33 | 24 | 16 | 12 | 15 | 12 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Haliangiaceae;Haliangium | 2 | 3 | 0 | 2 | 1 | 0 | 1 | 4 | 4 | 3 | 3 | 3 | 12 | 4 | 12 | 7 | 47 | 59 | 40 | 31 | 30 | 96 | 46 | 36 | 105 | 92 | 77 | 53 | 32 | 74 | 39 | 36 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Multi-affiliation;Multi-affiliation | 25 | 10 | 15 | 9 | 9 | 3 | 30 | 14 | 9 | 14 | 14 | 16 | 24 | 18 | 12 | 59 | 2 | 4 | 3 | 2 | 7 | 3 | 4 | 6 | 3 | 3 | 5 | 1 | 6 | 2 | 4 | 4 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Nannocystaceae;Nannocystis | 1 | 2 | 0 | 1 | 0 | 1 | 2 | 1 | 59 | 68 | 57 | 90 | 17 | 9 | 29 | 14 | 37 | 38 | 58 | 68 | 17 | 74 | 17 | 38 | 11 | 7 | 9 | 23 | 8 | 7 | 7 | 17 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Nannocystaceae;unknown genus | 0 | 2 | 1 | 2 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 13 | 4 | 1 | 3 | 4 | 22 | 43 | 50 | 236 | 16 | 5 | 69 | 54 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Polyangiaceae;Aetherobacter | 0 | 0 | 0 | 0 | 1 | 1 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 16 | 6 | 6 | 7 | 23 | 1 | 5 | 44 | 40 | 39 | 13 | 11 | 38 | 0 | 10 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Polyangiaceae;Sorangium | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 11 | 4 | 7 | 0 | 1 | 0 | 1 | 121 | 72 | 80 | 31 | 0 | 2 | 1 | 3 |
| Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Sandaracinaceae;Sandaracinus | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 39 | 32 | 22 | 28 | 15 | 4 | 20 | 19 | 36 | 22 | 29 | 41 | 33 | 20 | 59 | 38 | 59 | 44 | 55 | 82 | 34 | 42 | 41 | 66 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Gammaproteobacteria Incertae Sedis;unknown family;Permianibacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 14 | 5 | 3 | 1 | 15 | 2 | 7 | 9 | 60 | 21 | 17 | 1 | 22 | 8 | 22 | 16 | 15 | 11 | 10 | 1 | 20 | 5 | 20 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Perlucidibaca | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 69 | 122 | 53 | 51 | 1 | 0 | 0 | 1 | 83 | 110 | 81 | 60 | 1 | 1 | 1 | 4 | 47 | 42 | 36 | 38 | 1 | 1 | 0 | 1 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas | 36 | 11 | 13 | 6 | 3 | 4 | 11 | 8 | 141 | 69 | 126 | 136 | 57 | 60 | 49 | 125 | 120 | 109 | 147 | 119 | 52 | 77 | 59 | 122 | 111 | 116 | 114 | 113 | 50 | 88 | 58 | 124 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;unknown family;unknown genus | 11 | 8 | 7 | 6 | 17 | 12 | 22 | 15 | 4 | 6 | 4 | 4 | 11 | 13 | 25 | 9 | 8 | 5 | 6 | 5 | 16 | 17 | 21 | 16 | 6 | 7 | 9 | 6 | 21 | 19 | 27 | 18 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Arenimonas | 21 | 27 | 37 | 34 | 22 | 13 | 22 | 16 | 433 | 392 | 274 | 282 | 113 | 118 | 119 | 168 | 295 | 237 | 207 | 238 | 48 | 92 | 77 | 138 | 161 | 139 | 147 | 146 | 49 | 78 | 67 | 114 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Luteimonas | 11 | 5 | 14 | 3 | 10 | 7 | 20 | 6 | 108 | 132 | 105 | 101 | 229 | 134 | 179 | 208 | 203 | 168 | 163 | 169 | 184 | 138 | 186 | 229 | 100 | 134 | 128 | 138 | 114 | 130 | 134 | 166 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Lysobacter | 44 | 40 | 49 | 20 | 31 | 10 | 32 | 13 | 361 | 299 | 212 | 233 | 167 | 208 | 193 | 240 | 271 | 246 | 218 | 262 | 102 | 166 | 149 | 166 | 226 | 235 | 224 | 180 | 166 | 274 | 247 | 175 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Multi-affiliation | 2 | 3 | 4 | 2 | 2 | 6 | 1 | 1 | 445 | 340 | 545 | 690 | 20 | 66 | 34 | 42 | 236 | 136 | 213 | 278 | 15 | 47 | 20 | 39 | 83 | 129 | 78 | 414 | 21 | 57 | 41 | 44 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;Pseudoxanthomonas | 0 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 2 | 5 | 212 | 107 | 127 | 168 | 2 | 2 | 2 | 2 | 102 | 40 | 65 | 86 | 2 | 4 | 1 | 6 | 36 | 28 | 28 | 44 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadales Incertae Sedis;Acidibacter | 41 | 54 | 52 | 57 | 74 | 47 | 69 | 72 | 12 | 14 | 9 | 8 | 30 | 36 | 48 | 28 | 14 | 13 | 12 | 13 | 37 | 49 | 44 | 25 | 14 | 12 | 19 | 13 | 43 | 35 | 50 | 24 |
| Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadales Incertae Sedis;Multi-affiliation | 19 | 12 | 29 | 44 | 77 | 30 | 57 | 55 | 13 | 17 | 9 | 13 | 32 | 43 | 43 | 25 | 11 | 13 | 11 | 14 | 31 | 26 | 34 | 16 | 13 | 11 | 13 | 12 | 19 | 19 | 28 | 17 |

*Sup. data table 8: Abundance of microorganisms generas in different treatments, soils and sampling times (in number of cDNA sequences). The genera for which there were lasting significant differences between the two soils, or for which there were differences between treatments in garden soil (lasting or not), are shown in yellow (results of non-parametric ANOVA). The shades of red highlight these differences in abundance.*

## References

At the end of the manuscript

# Chapitre IV

# TRANSFERT TROPHIQUE DE L'Ag

Les données de la bibliographie ainsi que les données des expérimentations réalisées auparavant indiquent que l'argent, même sulfuré, reste légèrement biodisponible pour les organismes du sol, animaux et végétaux, et peut être bioaccumulé. En effet, le cloporte *Porcellio scaber* (Kampe et al., 2018), les vers de terre *E. andrei* (Velicogna et al., 2017) et *E. fetida* (Lahive et al., 2017) ont pu accumulé jusque 10 mg $kg^{-1}$ d'Ag (masses fraîche chez le cloporte ; sèche chez les vers de terre) dans leur organisme alors que l'Ag était apporté dans l'environnement sous forme sulfurée à des concentrations comprises entre 10 et 94 mg $kg^{-1}$. De même, chez les végétaux, en conditions réalistes, des bioaccumulations jusque 32 et 16 mg $kg^{-1}$ (m.s.) ont été observées respectivement dans les racines du blé et du colza lorsque le sol contenait 50-60 mg $kg^{-1}$ (Pradas del Real et al., 2016), sans que de l'Ag soit détecté dans leurs feuilles. Chez le jonc épars, le carex luisant et le panic érigé, des concentrations en Ag bioaccumulé allant jusque 0.2 mg $kg^{-1}$ ont été observées pour des concentrations dans le sol inférieures à 0.1 mg $kg^{-1}$ (Colman et al., 2013).

Cependant, en plus de la rareté des études, nous n'avons pas idée de son transfert potentiel le long d'une chaîne trophique. À ce jour, aucune étude n'a tenté de bâtir une petite chaîne alimentaire expérimentale afin d'évaluer le transfert de l'Ag sulfuré du sol vers les végétaux puis vers des invertébrés vivant à la surface du sol ou sur le couvert végétal. Dans ce contexte, nous avons décidé de mettre en place une expérimentation qui consistait en l'exposition de plusieurs espèces végétales à un sol mélangé avec une boue d'épuration contenant de l'Ag sulfuré, puis en la sélection d'une espèce végétale accumulatrice d'Ag afin de pouvoir la cultiver à plus grande échelle dans ce même substrat mais cette fois en subissant

une pression de pâturage par des escargots et des criquets. Ces 2 phases nous ont permis dans un premier temps de comparer les capacités d'accumulation de divers cultivars végétaux vis-à-vis de l'Ag sulfuré, et dans un second temps d'évaluer la capacité de transfert de cet Ag d'une espèce végétale vers des consommateurs primaires, tout en surveillant les traits de vie classiques chez les végétaux et animaux.

Cette expérimentation est décrite, analysée et discutée dans l'article qui suit intitulé « Transfer of sulfidized silver from silver nanoparticles, in sewage sludge, nanoparticles to plants and primary consumers in soil environment. ». Les résultats ont montré que l'$Ag_2S$ apporté par une boue peut s'accumuler chez les végétaux, rapidement mais avec une variabilité assez importante espèce dépendante. Les criquets et escargots qui consommaient uniquement l'espèce végétale accumulatrice n'ont pas significativement bioaccumulé d'Ag durant le temps d'exposition (2 et 5 semaines respectivement). Néanmoins, les escargots qui avaient eu accès au sol ont bioaccumulé tout de même de l'Ag, probablement par transfert cutanée et consommation directe de sol. Ainsi, bien que la spéciation de l'Ag dans les boues permette une moindre biodisponibilité du métal dans le sol pour les végétaux et animaux, cette pratique d'épandage des boues d'épuration sur les parcelles peut rester une source potentielle de contamination des chaînes alimentaires, et l'Homme peut être un consommateur principal du fait de la consommation des cultures.

# Transfer of sulfidized silver from silver nanoparticles, in sewage sludge, nanoparticles to plants and primary consumers in soil environment.

**Pauline Courtois**[1], Annette de Vaufleury[2], Anna Grosser[3], Christine Lors[4], and Franck Vandenbulcke[1]*

[1] *Univ. Lille, Institut Mines-Télécom, Univ. Artois, Junia, ULR 4515 - LGCgE - Laboratoire de Génie Civil et géo-Environnement, F-59000 Lille, France*

[2] *University of Bourgogne Franche-Comté, Department Chrono-environnement, UMR UFC/CNRS 6249, 16 route de Gray, Besançon Cedex, 25030, France*

[3] *Częstochowa University of Technology, Faculty of Infrastruture and Environment, Czestochowa, Poland*

[4] *IMT Lille Douai, Institut Mines-Télécom, Univ. Lille, Centre for Materials and Processes, F-59000 Lille, France*

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**Abstract**

Consumer products containing silver nanoparticles (AgNPs) release silver (Ag) to the environment, particularly wastewater. Sewage sludge (SS), which contains numerous contaminants including Ag, is recycled by spreading on agricultural land. Although slight impacts and bioaccumulation of Ag sulfide ($Ag_2S$, the main species found in SS) in terrestrial organisms have been demonstrated, possible trophic transfer into plants and subsequently animal species has not been examined. Accordingly, the present study experimentally measured the transfer of Ag from AgNPs and sulfidized Ag into plants and primary consumers and compared their bioavailability. Nine plant cultivars were grown in soil mixed with SS containing Ag, which revealed that bioaccumulation of Ag by plants is species-dependent. Ryegrass (the plant species with the greatest accumulation – up to 0.2 mg $kg^{-1}$) was then cultivated on a larger scale to expose snails and locusts for several weeks. While locusts did not accumulate Ag after two weeks of exposure, snails exhibited Ag bioaccumulation after 5 weeks when soil was accessible. Sulfidized Ag derived from AgNPs were less available (bioaccumulation up to 2.5 mg $kg^{-1}$) than the Ag from the original AgNPs (bioaccumulation up to 15 mg $kg^{-1}$). This transfer potential of Ag could have consequences for food webs due to chronic exposure linked to SS spreading practices. This study shows that transformations of AgNPs in treatment plants attenuate but do not completely eliminate the risk of Ag to plant and animal species SS.

**Keywords**

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

**Highlights**

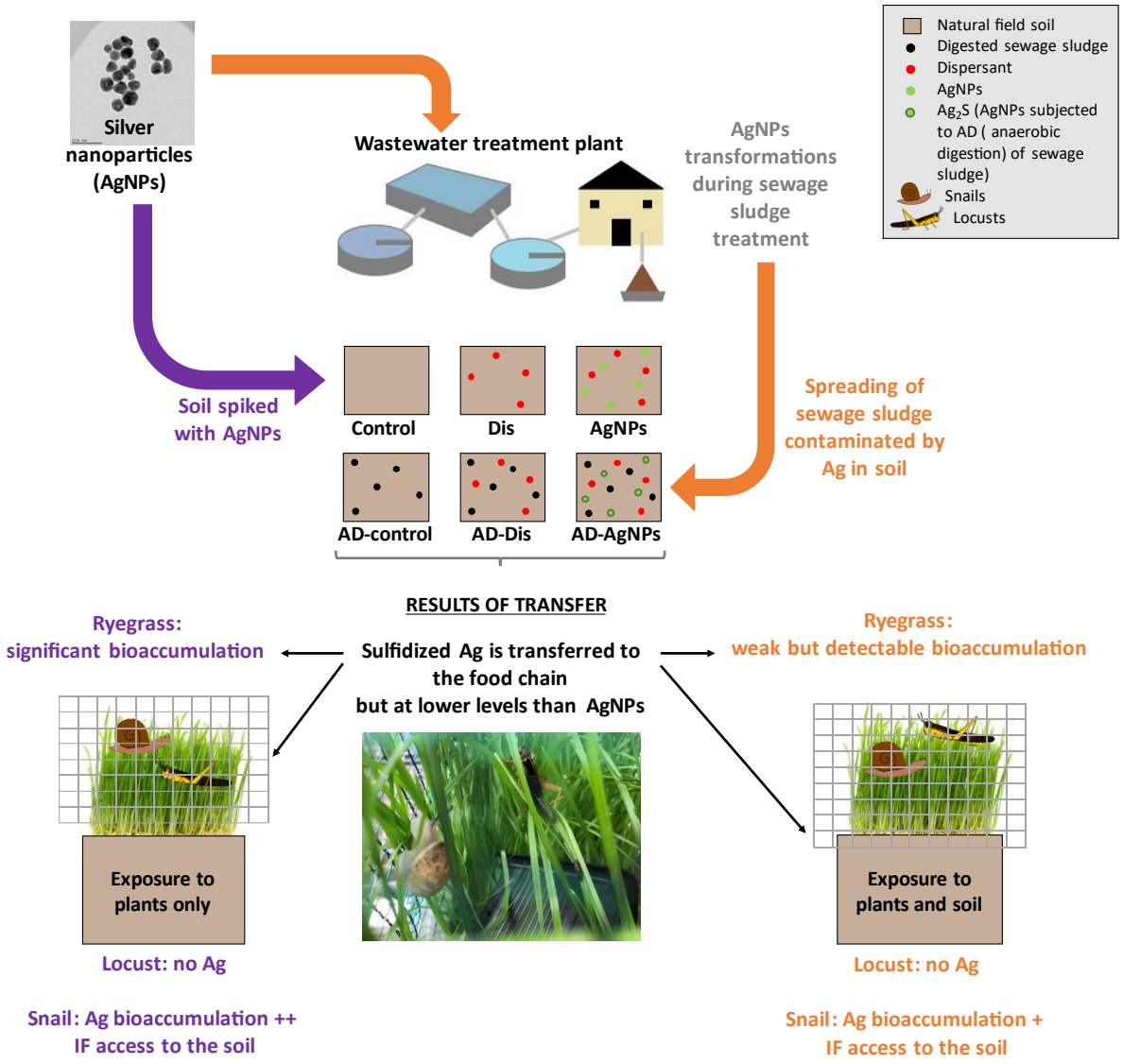
- Bioaccumulation of Ag by plants was species-dependent.

- Snails accumulated Ag when they can access contaminated soil.

- Sulfidized Ag were less bioavailable than manufactured Ag nanoparticles.

- Sulfidized Ag accumulated in the studied trophic chain during chronic exposure.

## Graphical abstract



## 1. Introduction

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

In response to these practices, the toxicity and ecotoxicity of Ag have been widely studied. Most of these works have studied Ag ions or intact AgNPs but it is now known that AgNPs released in wastewater undergo transformations in sewers and during the biosolid treatment (Levard et al., 2012; Ma et al., 2014; Pradas del Real et al., 2016). Indeed, AgNPs and $Ag^+$ ions can react with sulfur to give silver sulfide ($Ag_2S$) (Sekine et al., 2015). It is precisely $Ag_2S$ that is the predominant Ag species in SS, which has different properties than AgNPs or $Ag^+$ (Levard et al., 2013). Another previous study showed that the speciation of Ag after anaerobic digestion was $Ag_2S$ and Ag linked to thiols, that is to say two sulfidized forms of silver (Courtois et al., 2020). These Ag species have received less attention, and studies using SS containing realistic amounts of sulfidized Ag are scarce (Courtois et al., 2019).

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

For the first time, the bioavailability and the toxicity of various forms of Ag to a plant species and 2 soil invertebrates will be compared in a trophic chain experiment in order to assess the transfer of sulfidized Ag ($Ag_2S$ and Ag linked to thiols) from soil to plants and two complementary primary consumers. Small herbivorous invertebrates that are staple foods for many small carnivores and omnivores, including humans : the terrestrial snail *Cantareus aspersus* and the locust *Locusta migratoria* (Barker and Watts, 2002; Poma et al., 2017). These two species are complementary: one is a soft body invertebrate in close contact with soil, whereas the other has a cuticle and spends most of its life on plant cover. During this experiment, the Ag content in soil, plants and animals was quantified and vital parameters such as biomass and survival were monitored.

## 2. Materials and methods

### *2.1 Soil*

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

### *2.2 Sewage sludge*

Four wastewater treatment plants (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 in metals (silver, arsenic, cadmium, copper, manganese, nickel, lead) 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

$m^3$/d, population equivalents: 20,000). The metal content of the dry SS was as follows: 11.53 ± 1.43 mg $kg^{-1}$ Ag, 0.95 ± 0.22 mg $kg^{-1}$ arsenic (As), 1.10 ± 0.24 mg $kg^{-1}$ Cd, 140.62 ± 22.55 mg $kg^{-1}$ chromium (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, according to the method described in 2.7.2.). Before agricultural reuse, anaerobic stabilization of the SS was performed at laboratory scale.

### *2.3 Silver species*

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

A solution of $AgNO_3$ was prepared by dissolving $AgNO_3$ powder in milliQ water, taking into account the molar mass of each atom so that the concentration obtained corresponds well to a concentration of Ag and not of $AgNO_3$.

These two solutions of nanoparticles and silver ions were diluted with milliQ water to obtain Ag concentrations of approximately 2 mg $ml^{-1}$.

### *2.4 Plant species*

#### *2.4.1 Preliminary experiment*

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

#### *2.4.2 Main experiment*

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

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

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

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

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

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

| | Ag | As | Cd | Co | Cr | Cu | Hg | Mn | Mo | Ni | Pb | Sb | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. aspersus* | <LOQ | 0.828 ± 0.129 | 0.760 ± 0.015 | 1.100 ± 0.084 | 1.828 ± 0.136 | 152.836 ± 42.231 | 0.058 ± 0.100 | 92.208 ± 14.066 | 0.640 ± 0.022 | 1.924 ± 0.042 | <LOQ | 0.013 ± 0.022 | 310.978 ± 37.828 |
| *L. migratoria* | <LOQ | 0.825 ± 0.122 | 0.045 ± 0.041 | 0.043 ± 0.038 | 1.622 ± 1.540 | 44.001 ± 2.573 | 0.024 ± 0.042 | 6.688 ± 1.600 | 1.357 ± 0.865 | 1.116 ± 0.865 | 0.128 ± 0.201 | <LOQ | 130.064 ± 10.127 |

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

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

A batch anaerobic digestion (AD) of SS was performed in four continuous stirred-tank bioreactors at the beginning of 2017. These 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 analysis of pH, volatile fatty acids, volatile solids, total solids and ammonium nitrogen were described previously (Grosser, 2017).

In one bioreactor, 6 L of SS was digested without any addition. In a second bioreactor, the SS was spiked with 240 mg of AgNPs NM300K. In a third bioreactor, the SS was spiked with the corresponding quantity of dispersant used to suspend the AgNPs, and in a fourth bioreactor, the

SS was spiked with 240 mg of $AgNO_3$. The chosen Ag concentration (40 mg $L^{-1}$) in the bioreactors is justified in Supplemental information 1.

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

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

*2.6.2. Experimental design of preliminary exposure*

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

The 9 plant cultivars (of which two cultivars were the same species) were grown in a mixture of the agricultural soil and SS digested with $AgNO_3$. The mixture contained 19.9 kg of agricultural fresh soil (water content: 18.3 %) and 1.27 kg of thawed SS (water content 76.7 %) (i.e. 60 g of SS per kg of final mixture, justification in Sup. Inf. 2). The mixture was stirred frequently each day for 7 days and sifted immediately before distribution into boxes to ensure a relatively powdery soil facilitating seed germination. $AgNO_3$, like AgNPs, can react with sulfur and give $Ag_2S$ and Ag linked to thiols molecules. In a previous experiment, we were able to verify that the $Ag^+$ ions added to sewage sludge did indeed transform into $Ag_2S$ and Ag linked to thiols molecules during anaerobic fermentation (Courtois et al., 2020).

Each plant cultivar was sown in 3 boxes to establish triplicates. Each box (17 cm x 13 cm x 5 cm) contained 740 g of fresh soil mixture. An identical number of seeds was sown in each of

the 3 replicate boxes, but this number varied according to the characteristics of the species, specifically, plant size and acceptable plant density (Sup. Inf. 3).

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

*2.6.3. Experimental design of main exposure*

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

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

(b) 3 with digested SS: SS only ("AD-control"), SS with dispersant ("AD-dis"), and SS with AgNPs ("AD-AgNPs").

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

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

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

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

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

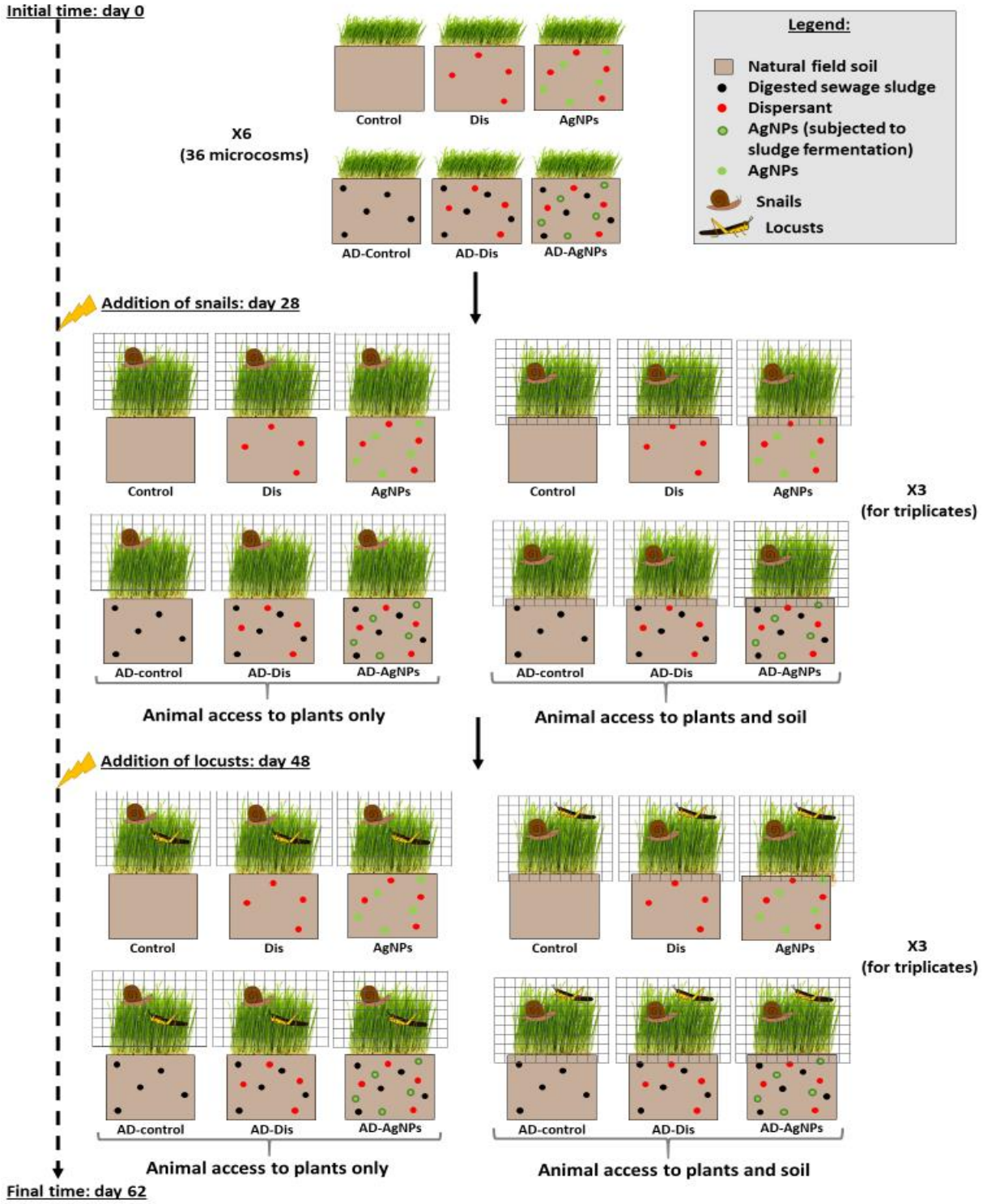



*Figure 1: Scheme of the main experimental design. The "Control", "Dis" and "AgNPs" conditions correspond to soil only, soil mixed with dispersant solution or soil mixed with AgNPs NM300K solution, respectively. The "AD-control" condition corresponds to soil mixed with sewage sludge digested without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

### *2.7. Analyses*

*2.7.1 Life traits of plants and animals*

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

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

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

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

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

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

For both experiments (preliminary and main), soil samples were collected at the beginning of exposure and dried at 80 °C until total dehydration (stabilization of the weight). The dried samples were ground with a mortar and pestle.

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

snails) and at 62 days (final time) of exposure. The leaves were rinsed several times with milli Q water, wiped, dried in an oven at 40 °C until total dehydration and crushed.

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

For the preliminary experiment, soil mineralization was performed by digesting 500 mg of soil in aqua regia ($HNO_3$:HCl 2:5, v:v ultratraces, Optima®, Fisher Scientific, Illkirch, France). The resulting solutions were analyzed by ICP-MS (inductively coupled plasma mass spectrometry, Thermo Scientific X Series II, Courtaboeuf, France) to quantify As, Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn; Ag was quantified by ICP-AES (inductively coupled plasma atomic emission spectroscopy, ICAP 6500 Radial de Thermo Scientific, Courtaboeuf, France). A certified material (loamy clay 1, CRM052, LGC Standards, Molsheim, France) was employed

the same day with both instruments for verification and gave recovery rates of 85 % to 120% for all metallic elements. For the main experiment, 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, Berghof, Eningen, Germany), and same metals were analyzed by ICP-OES (inductively coupled plasma optical emission spectrometry, Thermo Scientific, Courtaboeuf, France). The protocol used to analyze the metal contents in the sewage sludge monthly during the year 2016 was exactly the same than the one for the main experience.

For mineralization of plants and animals, digestion was performed in acid medium (using $HNO_3$, $H_2SO_4$ and $HCl_4$) at high temperature as described by Bernard et al. (2010). Lanier et al. (2019) showed that this method was effective for the reference organisms. The obtained solution was then used to quantify As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn and Ag by ICP-OES (Agilent Technologies 5110, USA, for the preliminary experiment and Varian 720-ES, USA, for the main one). Some additional metals were quantified only in the main experiment: Sb, Mo and Hg. These metals are conventionally analyzed in foodstuffs intended for humans and animals (Directive 2002/32 of European Commission, 2002; Regulation n° 1881/2006 of European Commission, 2006) or in the context of ecotoxicological analyses. The effectiveness of the mineralization was verified and validated by mineralizing reference materials (IC-INCT-OBTL-5 from LabMix24 Germany for plants and TORT-3 and DOLT-5 from National Research Council Canada for animals).

### *2.7.3 Statistical analyses*

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

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

## 3. Results

### *3.1. Preliminary experiment*

*Metal content in soil*

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

*Life traits of plants*

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

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

*Metal accumulation in plants*

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

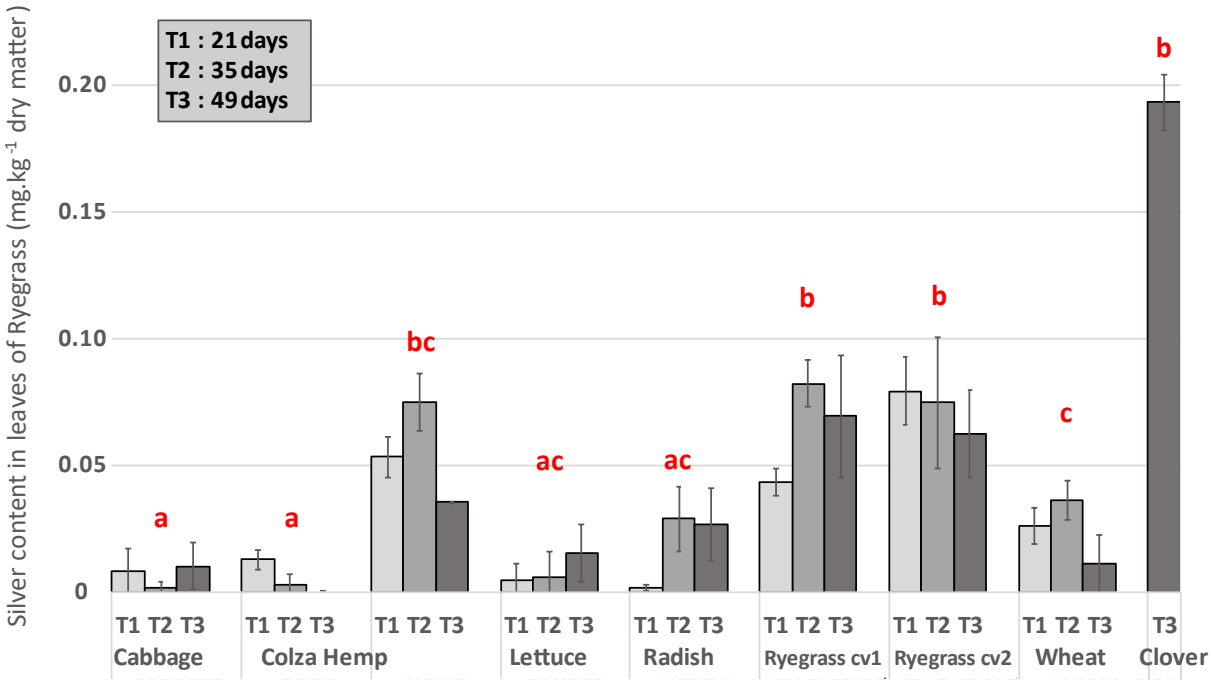



*Figure 2: Silver concentration in leaves of plants in mg $kg^{-1}$ (dry matter). Significant differences are indicated by different letters (comparison between species only, data from all time points pooled since there were no significant differences among the 3 time points for each species). T1, T2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

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

### *3.2. Main experiment*

*Metal contents in mesocosm mixtures*

In general, there was little difference in metal concentrations among the 6 mesocosm conditions (Table 2). Zn content was higher in the conditions with SS than in those without. Ag content was significantly higher in the conditions with AgNPs or AD-AgNPs than in Control and Disor AD-control and AD-dis, respectively. However, the Ag content was significantly lower in AD-AgNPs than in the AgNPs mesocosms (almost two times less). It is therefore important to carefully interpret the comparisons of the AgNPs and AD-AgNPs conditions.

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

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

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

*Plant life traits: leaf growth*

Ryegrass growth was observed in all mesocosms. From the second week, it appeared visually that the *Lolium perenne* grew much faster in the mesocosms with SS than those without SS

(Sup. Inf. 4). This visual difference in biomass was maintained until the end of the experiment. Visually, there was therefore no effect of Ag on the length and the biomass of ryegrass; only the effect of SS was visible.

*Animal life traits: survival and biomass*

Snail and locust survival were not correlated with condition or soil access according to the statistical tests (non-parametric ANOVA) (Table 4).

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

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

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

| | | | | | |
|---|---|---|---|---|---|
| | P+S | 83.33 ± 15.28 | + 0.04 ± 0.07 | 66.67 ± *23.09* | + 0.09 ± 0.09 |
| AD-AgNPs | P | 66.67 ± 23.09 | - 0.16 ± 0.16 | 46.67 ± *23.09* | + 0.12 ± 0.21 |
| | P+S | 76.67 ± 15.28 | + 0.32 ± 0.14 | 60.00 ± *20.00* | + 0.03 ± 0.17 |
| *Average* | | *80.28 ± 15.94* | *- 0.02 ± 0.21* | *61.11 ± 24.82* | *+ 0.04 ± 0.13* |

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

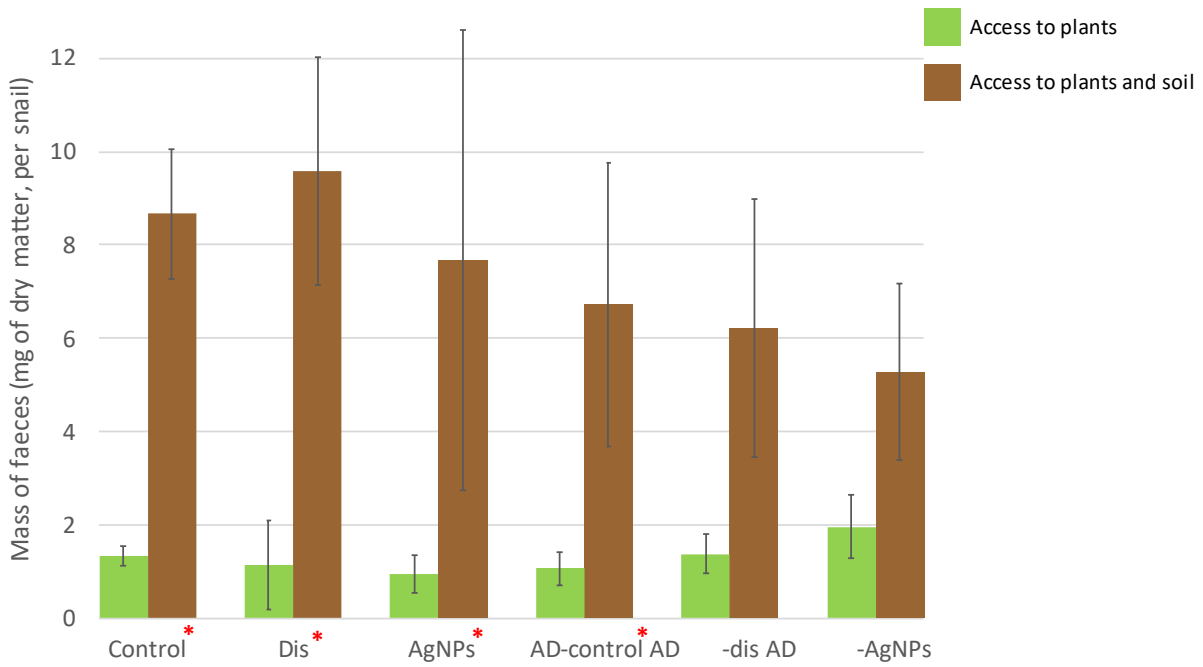



*Figure 3: Quantity of faeces produced by snails during the final fasting depuration in mg of dry matter. Red stars indicate significant differences between 'access to plants' and 'access to plants and soil' within the same condition.*

*Metal accumulation in plants*

Consistent with the results of the preliminary experiment, ryegrass accumulated Ag when cultivated in soil enriched with Ag (Figure 4 A). In the condition without SS (AgNPs), the Ag content in the aerial parts of ryegrass was very significant (mean of 0.68 mg $kg^{-1}$ of Ag in dry matter). By comparison, in the AD-AgNPs condition, Ag was detectable in ryegrass, but the content was lower (0.02 mg $kg^{-1}$ in mean) and did not differ significantly from that in the mesocosm without Ag. These results indicated that the sulfidized Ag contained in the SS was less bioavailable to ryegrass than AgNPs. This conclusion was confirmed by the calculation of the bioaccumulation factor (BAF) (Sup. Inf. 8): the BAF for ryegrass was 0.061 in the AgNPs mesocosms but 0.003 in the AD-AgNPs mesocosms, a difference of 20-fold.

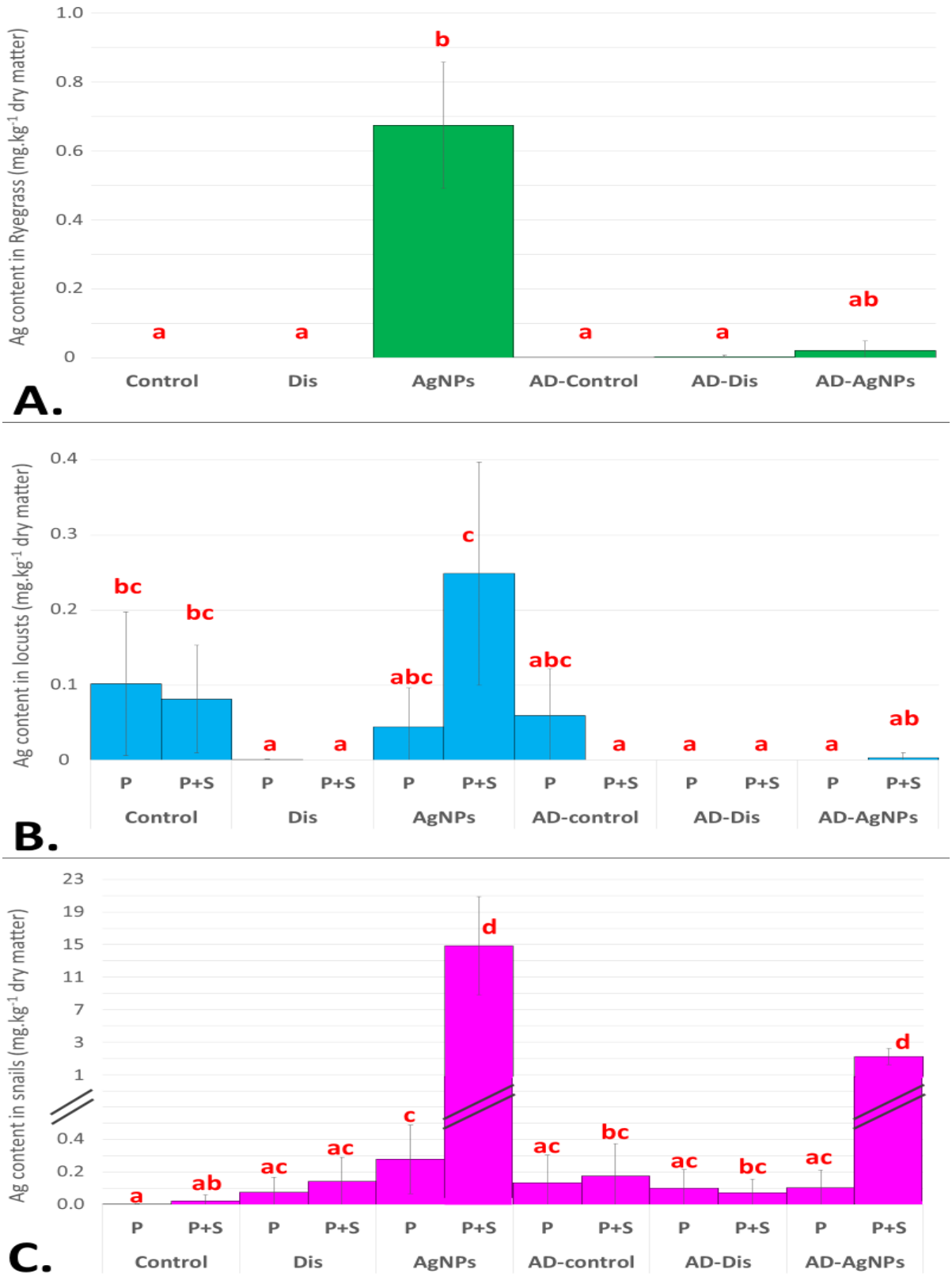



*Figure 4: Silver concentration in organisms at the end of experiment. A. Silver content in the aerial part of ryegrass in mg kg$^{-1}$ (dry matter). Significant differences are indicated by different letters. B. Silver content in whole locusts in mg kg$^{-1}$(dry matter). Significant differences are*

*indicated by different letters. C. Silver content in snails (without shell) in mg kg$^{-1}$(dry matter). Significant differences are indicated by different letters.*

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

*Metal accumulation in locusts.*

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

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

*Metal accumulation in snails.*

In contrast to the results observed in locusts, snails significantly bioaccumulated Ag under two conditions: AgNPs with soil and plant access, which yielded the highest concentrations (15.25 ± 5.73 mg kg$^{-1}$ of dry matter), and AD-AgNPs with soil and plant access (Figure 4 C). The BAF for AD-AgNPs with soil and plant access was 4 times lower than that

for AgNPs with soil and plant access (Sup. Inf. 7). In the other conditions with Ag without access to soil, the Ag content in snails was not different from that of snails that were not exposed to Ag.

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

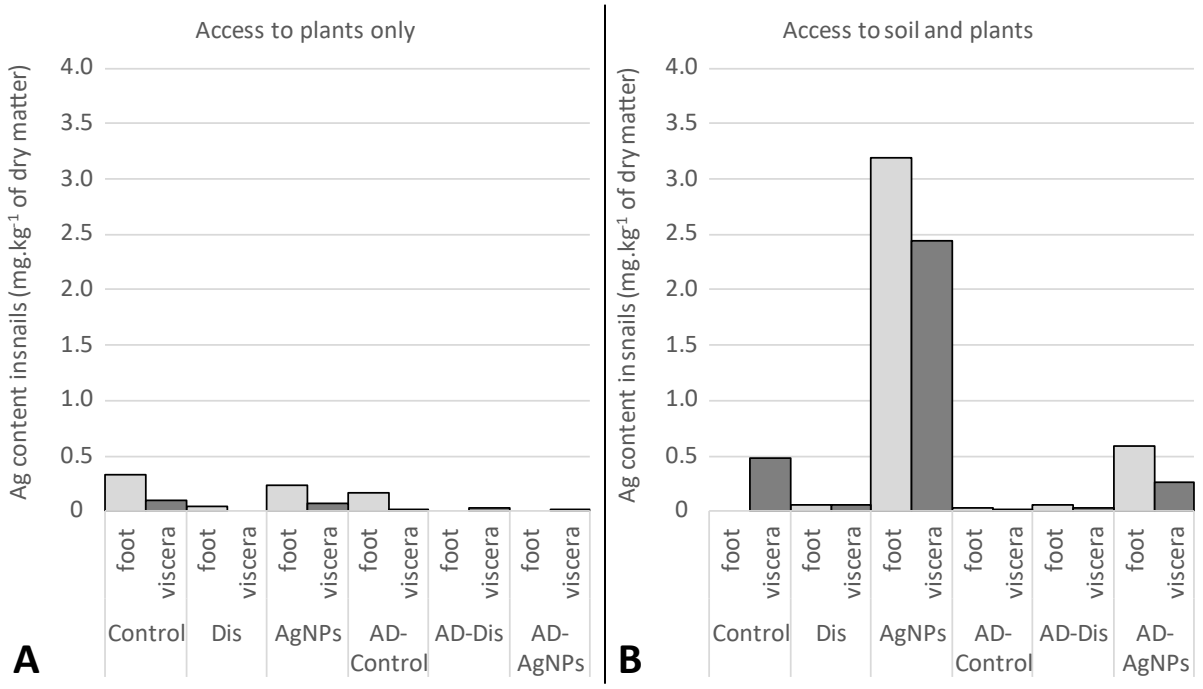



*Figure 5: Silver concentration (in mg $kg^{-1}$ of dry matter) in snail foot and viscera exposed to (A) to plants only and (B) to soil and plants. The absence of error bars and statistical comparisons is due to the impossibility of making replicates.*

For snails, the accumulation of Cu, Sb, As, Cr, Mn, Mo, Hg, Ni and Zn was similar in all conditions. However, the accumulated amount of Cd was significantly greater in snails that were in contact with soil than in snails without access to soil (Sup. Inf. 7). The average BAF for Cd was 5.99 for snails with plant+soil access but 3.33 for snails with plant access only (Sup.

Inf. 8). Similar effects were observed for Co and Pb, but the differences between the two types of access were not statistically significant.

## 4. Discussion and conclusion

*Transfer and effects of Ag on plants*

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

The beneficial effects of SS on plant growth and biomass have been known for several years (Kumar et al., 2017) and were confirmed in the main experiment on ryegrass. Ag did not visually affect the appearance and aerial biomass of this species, consistent with the observations of Pradas del Real et al. (2016). In their study, sulfidized Ag affected the root growth of rape and wheat even with Ag concentrations around 1 mg $kg^{-1}$. Moreover, our results

confirmed the lower bioavailability of sulfidized Ag compared with AgNPs previously reported in the literature (Doolette et al., 2015; Pradas del Real et al., 2017, 2016; Stegemeier et al., 2015; Wang et al., 2015), since the bioaccumulation of sulfidized Ag by ryegrass was approximately 20 times lower than that of AgNPs according to the BAF calculations. Indeed, $Ag_2S$ is very poorly soluble compared with AgNPs (Levard et al., 2013; Pradas del Real et al., 2017), which reduces the release of $Ag^+$ ions that can easily penetrate plant cells, particularly since the Ag added to SS before anaerobic digestion persists for several weeks in sulfidized form in soil (according to a study using the same soil, SS from the same treatment plant, and similar anaerobic digestion with identical Ag concentrations; Courtois et al., 2020).

*Transfer and effects of Ag on soil invertebrates*

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

While locusts did not significantly accumulate Ag, their period of exposure was shorter than that of snails. Studies have shown that for snails, 2 weeks is sufficient to observe significant bioaccumulation of Cd (Coeurdassier et al., 2002; Gimbert et al., 2006), whereas 4 weeks is required for Ag, Cu, Pb and Zn (De Vaufleury, 2015; Mariet et al., 2017). We can therefore conclude that the duration of exposure of snails in the present study (almost 5 weeks) was sufficient to allow the bioaccumulation of Ag and other metals, whereas 2 weeks of exposure of locusts may not have been sufficient to observe significant bioaccumulation of metals like Ag. Comparable data are not available for locusts because most studies recover animals from

the field and therefore the duration of exposure is unknown (Devkota and Schmidt, 2000; Soliman and El-Shazly, 2017; Zhang et al., 2012). This first reassuring result should therefore be confirmed by longer exposure of locusts. However, in addition to the short exposure time, the Ag levels in locusts may not be detectable due to the low Ag levels in plants.

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

amount of Ag still transferred via the food chain. A comparative tomographic analysis of snails in contact with plants only and those previously in contact with soil might reveal whether part of the Ag is adsorbed on the foot or if all of it is passed into the tissues of the snail. Such an analysis would also identify the main sites of bioaccumulation of Ag in this snail species.

In the most realistic condition (AD-AgNPs with access to soil), the snails accumulated approximately 2.3 mg $kg^{-1}$ of Ag, compared with 15 mg $kg^{-1}$ in the same condition with AgNPs. These contents are clearly higher than those found in a study by Mariet et al. (2017) that examined the bioaccumulation of Ag in different areas of an old lead-silver mine. In Mariet et al. (2017), the Ag concentration reached 1.14 mg kg-1 in the digestive gland (a part of the viscera) of *Cantareus asperses* snails exposed to soil containing 14.5 mg $kg^{-1}$ of Ag (BAF: 0.054). Several factors may explain the lower transfer observed in this previous field study, for instance, soil characteristics, metal speciation in soil, reduced contact and eating of soil due to the humus cover and the possibility of eating various plants exhibiting different capacities of Ag accumulation. Regardless, our results are reassuring in the context of land spreading of SS and are consistent with previous studies of ryegrass and earthworms (Courtois et al., 2020 ; Velicogna et al., 2017): the Ag found in SS is less bioavailable than AgNPs due to its speciation. The higher organic matter content in soil supplemented with SS may have increased this bioaccumulation gap (between snails in AgNPs P+S and in AD-AgNPs P+S). In fact, the snails under AD-AgNPs conditions probably consumed fewer soil particles to meet their nutritional needs than those under AgNPs conditions, and therefore the latter also consumed more metals. In addition, it would be interesting to compare the speciation of Ag in plants grown under AgNPs conditions and AD-AgNPs conditions. Wang et al. (2015) and Pradas del Real et al. (2017) showed that the speciation of bioaccumulated Ag varied according to plant treatment. In these studies, $Ag_2S$-NPs treatment resulted in bioaccumulation of Ag mainly in $Ag_2S$ form, AgNPs treatment resulted in bioaccumulation of Ag mainly in metallic form and linked to a

thiol molecule, and $AgNO_3$ treatment resulted in bioaccumulation of Ag mainly linked to a thiol molecule. If $Ag_2S$ could enter plants via the roots, the bioaccumulated $Ag_2S$ might be less bioavailable, resulting in less bioaccumulation in animals.

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

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

attenuate but do not completely prevent the accumulation of Ag in plant and animal species. SS spreading is a common practice, and it may be possible to make informed decisions on the choice of plots to amend with SS according to the plant species planned for cultivation. Thus, it seems important to add Ag to the list of metals controlled in SS (European directive 86/278/CEE, 1986) in order to ensure that SS containing Ag is spread primarily on plots that will receive cultivated species that accumulate little Ag. When SS is too rich in Ag, other strategies for SS recycling, could be employed, such as remediation of polluted soil.

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The authors declare no competing financial interest.

**Supplemented informations**

*Sup. Inf. 1: Justification for choosing silver concentration in bioreactors.*

According to a report by U.S. EPA published in 2009, the average content of AgNPs in SS varies between 2 and 856 mg $kg^{-1}$. In this study, we decided to use a final concentration of 40 mg $L^{-1}$ in bioreactors containing a final volume of 6 L. Yang et al. (2013) investigated the possible impact of high doses of Ag on the anaerobic digestion process (anaerobic glucose degradation, SS digestion, methanogenic assemblages). They observed that high doses of Ag (up to 40 mg $L^{-1}$) did not significantly impact biogas and methane production or the anaerobic digestion process. Moreover, the authors reported that more than 90% of AgNPs were removed from the liquid phase. The concentration of the AgNPs NM-300K stock solution was 2 mg $L^{-1}$. Thus, 120 mL of this solution was added to the bioreactor receiving AgNPs to add 240 mg of Ag. A solution of $AgNO_3$ was prepared at the same concentration of Ag and the same quantity of solution was added to the bioreactor receiving $AgNO_3$. For the bioreactor receiving SS with dispersant, 120 mL of dispersant was added. The bioreactor with the control SS did not contain any additive.

*Sup. Inf. 2: Justification for choosing the sewage sludge doses in the microcosms*

The doses of SS introduced in the microcosms were chosen according to European legislation, which states that it is possible to apply up to 30 tons of dry matter per hectare for a period of 10 years (Directive européenne 86/278/CEE, 1986). The density of soil used in this study was 1.25 $g.cm^3$, and the average depth of incorporation of SS into agricultural soil was approximately 8 cm (typically 5 to 15 cm). Thus, the soil mass that is mixed with SS over one hectare corresponds to approximately 1000 tons (density = mass/volume). Therefore, from a one-year perspective, it is possible to apply 3 tons of SS (dry matter) per 1000 tons of soil, i.e.

3 g of SS per kg of soil. Assuming that dewatered SS still contains approximately 85% water, the mass allowed for spreading is close to 20 g of fresh SS per kg of soil per year. However, European legislation allows the addition, at one time, of significantly higher SS quantities on agricultural soil, since it is possible to add up to 30 tons of SS per hectare per decade. It is therefore legally possible to apply the equivalent of several years at one time, which allows the input of up to 200 g of fresh SS per kg of soil per decade.

*Sup. Inf. 3: Quantities of seeds used for preliminary experiment in each box.*

| Species | Number or mass of seeds per mesocosm |
|---|---|
| *Brassica napus* | 15 seeds |
| *Brassica oleracea* | 15 seeds |
| *Cannabis sativa* | 20 seeds |
| *Lactuca sativa* | 6 seeds |
| *Lolium perenne, calao* variety | 1 g |
| *Lolium perenne, bocage* variety | 1 g |
| *Raphanus sativus* | 15 seeds |
| *Trifolium repens* | 1 g |
| *Triticum aestivum* | 75 seeds |

*Sup. Inf. 4: Pictures of two microcosms in the main experiment at day 41. Plant development was similar in all microcosms with SS (A) but was reduced in microcosms without SS (B). This difference was apparent from the start of plant growth and lasted until the end of the experiment.*

*Sup. Inf. 5: Metals content in aerial part of Ryegrass in mg $kg^{-1}$ (dry matter) at the intermediate (4 weeks) and final time points (almost 9 weeks).*

| | Control | | Dis | | AgNPs | | AD-Control | | AD-Dis | | AD-AgNPs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | Intermediate | Final | Intermediate | Final | Intermediate | Final | Intermediate | Final | Intermediate | Final | Intermediate | Final |
| Antimony | 0.047 ± 0.075 | 0.001 ± 0.002 | 0.073 ± 0.164 | 0.035 ± 0.046 | 0.000 ± 0.000 | 0.040 ± 0.063 | 0.029 ± 0.072 | 0.000 ± 0.000 | 0.079 ± 0.123 | 0.008 ± 0.019 | 0.008 ± 0.019 | 0.007 ± 0.012 |
| Arsenic | 0.999 ± 0.526 | 0.884 ± 0.173 | 0.507 ± 0.221 | 0.877 ± 0.102 | 0.644 ± 0.509 | 0.942 ± 0.282 | 0.632 ± 0.184 | 0.948 ± 0.164 | 0.917 ± 0.381 | 0.979 ± 0.176 | 0.616 ± 0.327 | 0.889 ± 0.146 |
| Cadmium | 0.135 ± 0.070 | 0.069 ± 0.035 | 0.088 ± 0.110 | 0.058 ± 0.024 | 0.004 ± 0.011 | 0.018 ± 0.015 | 0.157 ± 0.067 | 0.055 ± 0.029 | 0.120 ± 0.056 | 0.074 ± 0.050 | 0.121 ± 0.073 | 0.050 ± 0.017 |
| Chromium | 0.000 ± 0.000 | 0.426 ± 0.228 | 0.288 ± 0.241 | 0.329 ± 0.561 | 0.310 ± 0.288 | 0.224 ± 0.204 | 0.296 ± 0.433 | 0.056 ± 0.070 | 0.603 ± 0.377 | 0.048 ± 0.060 | 0.391 ± 0.312 | 0.088 ± 0.048 |
| Cobalt | 0.094 ± 0.043 | 0.080 ± 0.027 | 0.315 ± 0.273 | 0.127 ± 0.029 | 0.596 ± 0.402 | 0.111 ± 0.049 | 0.943 ± 0.921 | 0.101 ± 0.035 | 2.175 ± 1.360 | 0.134 ± 0.038 | 0.193 ± 0.073 | 0.128 ± 0.044 |
| Copper | 8.757 ± 2.596 | 9.558 ± 1.184 | 7.018 ± 1.198 | 9.963 ± 0.487 | 4.140 ± 0.380 | 4.198 ± 1.270 | 12.366 ± 0.960 | 10.857 ± 0.960 | 12.355 ± 0.663 | 10.918 ± 0.618 | 11.702 ± 0.822 | 9.685 ± 0.287 |
| Manganese | 48.147 ± 6.581 | 54.135 ± 5.764 | 48.971 ± 9.955 | 50.073 ± 5.304 | 24.625 ± 5.100 | 45.130 ± 7.906 | 26.842 ± 3.465 | 18.392 ± 1.109 | 28.487 ± 1.682 | 17.637 ± 1.395 | 28.320 ± 6.138 | 18.571 ± 2.054 |
| Mercury | 0.000 ± 0.000 | 0.010 ± 0.025 | 0.000 ± 0.000 | 0.003 ± 0.005 | 0.031 ± 0.075 | 0.000 ± 0.000 | 0.010 ± 0.024 | 0.017 ± 0.035 | 0.005 ± 0.013 | 0.015 ± 0.020 | 0.000 ± 0.000 | 0.002 ± 0.004 |
| Molybdene | 2.095 ± 0.281 | 2.636 ± 0.393 | 1.987 ± 0.473 | 2.516 ± 0.437 | 1.450 ± 0.353 | 2.470 ± 0.451 | 1.562 ± 0.190 | 1.168 ± 0.280 | 1.613 ± 0.406 | 1.144 ± 0.166 | 1.863 ± 0.609 | 1.391 ± 0.173 |
| Nickel | 1.331 ± 0.279 | 1.038 ± 0.096 | 1.226 ± 0.281 | 1.019 ± 0.124 | 0.941 ± 0.299 | 0.862 ± 0.086 | 1.262 ± 0.349 | 0.583 ± 0.045 | 1.030 ± 0.176 | 0.613 ± 0.084 | 0.987 ± 0.117 | 0.661 ± 0.088 |
| Lead | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| Zinc | 49.778 ± 4.556 | 69.534 ± 7.384 | 53.581 ± 0.091 | 50.453 ± 6.143 | 37.850 ± 5.128 | 44.162 ± 5.913 | 78.241 ± 11.056 | 64.652 ± 8.847 | 75.925 ± 7.977 | 51.426 ± 3.363 | 79.656 ± 7.378 | 53.919 ± 4.946 |

*Sup. Inf. 6: Metal contents in locusts in mg kg$^{-1}$ (dry matter) at the final time point. The "P" and "P+S" accesses mean that animals had access to plants only or to plants and soil, respectively.*

| Condition | Control | | Dis | | AgNPs | | AD-Control | | AD-Dis | | AD-AgNPs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Access | P | P+S | P | P+S | P | P+S | P | P+S | P | P+S | P | P+S |
| Antimony | 0.009 ± 0.028 | 0.016 ± 0.046 | 0.000 ± 0.000 | 0.014 ± 0.026 | 0.017 ± 0.038 | 0.017 ± 0.032 | 0.003 ± 0.009 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.001 ± 0.004 | 0.011 ± 0.027 | 0.000 ± 0.000 |
| Arsenic | 0.852 ± 0.261 | 0.986 ± 0.377 | 0.787 ± 0.271 | 0.888 ± 0.233 | 0.608 ± 0.301 | 0.519 ± 0.237 | 0.985 ± 0.300 | 0.850 ± 0.447 | 0.865 ± 0.201 | 0.745 ± 0.254 | 0.560 ± 0.348 | 0.453 ± 0.352 |
| Cadmium | 0.005 ± 0.009 | 0.015 ± 0.030 | 0.035 ± 0.068 | 0.016 ± 0.025 | 0.009 ± 0.012 | 0.039 ± 0.064 | 0.030 ± 0.027 | 0.030 ± 0.034 | 0.009 ± 0.024 | 0.026 ± 0.034 | 0.022 ± 0.036 | 0.018 ± 0.022 |
| Chromium | 3.518 ± 1.132 | 1.688 ± 0.726 | 4.471 ± 1.961 | 8.005 ± 7.685 | 8.739 ± 5.431 | 10.949 ± 6.474 | 3.616 ± 2.173 | 7.997 ± 4.897 | 7.483 ± 5.093 | 11.457 ± 9.605 | 7.080 ± 3.804 | 12.849 ± 3.181 |
| Cobalt | 0.099 ± 0.071 | 0.086 ± 0.044 | 0.097 ± 0.078 | 0.171 ± 0.198 | 0.256 ± 0.190 | 0.316 ± 0.190 | 0.059 ± 0.052 | 0.216 ± 0.143 | 0.283 ± 0.335 | 0.235 ± 0.118 | 0.202 ± 0.148 | 0.174 ± 0.145 |
| Copper | 46.883 ± 5.150 | 47.312 ± 5.645 | 45.605 ± 4.174 | 49.646 ± 2.850 | 44.800 ± 5.263 | 47.086 ± 4.626 | 46.295 ± 3.197 | 45.737 ± 2.889 | 47.821 ± 5.296 | 46.891 ± 4.914 | 46.299 ± 4.499 | 48.107 ± 2.123 |
| Manganese | 4.152 ± 1.292 | 4.516 ± 1.086 | 3.937 ± 1.194 | 4.152 ± 1.340 | 3.852 ± 1.013 | 6.986 ± 1.520 | 2.501 ± 0.534 | 3.651 ± 1.105 | 2.678 ± 0.810 | 4.444 ± 1.594 | 3.070 ± 0.977 | 4.945 ± 1.381 |
| Mercury | 0.186 ± 0.129 | 0.090 ± 0.140 | 0.076 ± 0.142 | 0.033 ± 0.051 | 0.017 ± 0.043 | 0.080 ± 0.096 | 0.096 ± 0.108 | 0.055 ± 0.090 | 0.095 ± 0.144 | 0.062 ± 0.100 | 0.055 ± 0.114 | 0.034 ± 0.064 |
| Molybdene | 1.091 ± 0.369 | 0.959 ± 0.337 | 0.816 ± 0.354 | 0.757 ± 0.315 | 0.958 ± 0.359 | 0.769 ± 0.317 | 0.880 ± 0.363 | 0.587 ± 0.378 | 0.586 ± 0.331 | 0.477 ± 0.140 | 0.673 ± 0.416 | 0.815 ± 0.303 |
| Nickel | 2.416 ± 0.656 | 1.435 ± 0.665 | 2.297 ± 1.161 | 3.359 ± 3.415 | 4.171 ± 2.258 | 5.043 ± 2.797 | 2.262 ± 0.960 | 3.678 ± 2.115 | 3.161 ± 2.234 | 4.868 ± 4.002 | 3.391 ± 1.869 | 6.160 ± 1.526 |
| Lead | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| Zinc | 161.900 ± 23.454 | 161.405 ± 10.602 | 157.576 ± 22.056 | 170.054 ± 16.393 | 149.192 ± 11.830 | 155.471 ± 11.150 | 159.550 ± 18.124 | 162.827 ± 15.119 | 150.627 ± 16.211 | 156.512 ± 13.318 | 159.200 ± 11.127 | 167.316 ± 13.665 |

*Sup. Inf. 7: Metal contents in snails in mg $kg^{-1}$ (dry matter) at final time point. The "P" and "P+S" accesses mean that animals had access to plants only or to plants and soil, respectively.*

| Condition | Control | | Dis | | AgNPs | | AD-Control | | AD-Dis | | AD-AgNPs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Access | P | P+S | P | P+S | P | P+S | P | P+S | P | P+S | P | P+S |
| Antimony | 0.048 ± 0.116 | 0.068 ± 0.108 | 0.188 ± 0.171 | 0.115 ± 0.116 | 0.160 ± 0.173 | 0.112 ± 0.116 | 0.145 ± 0.123 | 0.091 ± 0.095 | 0.197 ± 0.185 | 0.131 ± 0.220 | 0.086 ± 0.122 | 0.081 ± 0.103 |
| Arsenic | 0.537 ± 0.392 | 0.758 ± 0.443 | 0.777 ± 0.322 | 0.967 ± 0.273 | 0.705 ± 0.425 | 0.871 ± 0.418 | 0.798 ± 0.300 | 0.625 ± 0.325 | 0.845 ± 0.355 | 0.686 ± 0.322 | 0.838 ± 0.215 | 0.853 ± 0.345 |
| Cadmium | 1.103 ± 0.180 | 2.029 ± 0.309 | 1.140 ± 0.069 | 2.092 ± 0.510 | 0.910 ± 0.123 | 1.875 ± 0.402 | 1.015 ± 0.169 | 1.517 ± 0.363 | 1.089 ± 0.257 | 1.688 ± 0.236 | 0.942 ± 0.114 | 1.951 ± 0.233 |
| Chromium | 5.586 ± 1.790 | 4.377 ± 1.140 | 1.205 ± 0.333 | 1.190 ± 0.521 | 1.647 ± 0.953 | 3.424 ± 1.750 | 1.518 ± 0.446 | 1.864 ± 0.530 | 0.934 ± 0.450 | 1.576 ± 0.267 | 1.853 ± 0.550 | 2.898 ± 0.957 |
| Cobalt | 0.791 ± 0.165 | 1.290 ± 0.366 | 0.772 ± 0.299 | 1.085 ± 0.813 | 0.702 ± 0.246 | 1.131 ± 0.310 | 0.649 ± 0.189 | 0.841 ± 0.276 | 0.472 ± 0.101 | 0.739 ± 0.140 | 0.515 ± 0.253 | 0.848 ± 0.316 |
| Copper | 155.270 ± 26.010 | 160.638 ± 33.159 | 156.756 ± 49.843 | 175.321 ± 41.411 | 164.732 ± 27.128 | 164.620 ± 28.842 | 144.943 ± 18.496 | 136.729 ± 37.534 | 131.262 ± 27.899 | 159.317 ± 21.345 | 154.122 ± 21.097 | 163.141 ± 31.439 |
| Manganese | 91.081 ± 16.026 | 126.693 ± 27.219 | 103.024 ± 21.887 | 118.003 ± 27.348 | 96.505 ± 23.031 | 159.259 ± 55.529 | 88.929 ± 13.916 | 88.109 ± 13.233 | 84.073 ± 13.400 | 87.378 ± 18.470 | 91.214 ± 13.873 | 90.427 ± 24.887 |
| Mercury | 0.000 ± 0.000 | 0.022 ± 0.049 | 0.102 ± 0.115 | 0.055 ± 0.046 | 0.041 ± 0.093 | 0.019 ± 0.056 | 0.116 ± 0.146 | 0.057 ± 0.087 | 0.124 ± 0.224 | 0.055 ± 0.072 | 0.000 ± 0.000 | 0.007 ± 0.015 |
| Molybdene | 0.821 ± 0.191 | 1.173 ± 0.458 | 0.825 ± 0.254 | 1.207 ± 0.354 | 0.798 ± 0.254 | 0.982 ± 0.243 | 1.221 ± 0.592 | 1.720 ± 0.394 | 0.913 ± 0.207 | 1.354 ± 0.287 | 1.550 ± 0.613 | 1.491 ± 0.373 |
| Nickel | 3.015 ± 0.772 | 2.680 ± 0.562 | 1.146 ± 0.463 | 1.036 ± 0,538 | 1.229 ± 0.438 | 2.003 ± 0.823 | 1.111 ± 0.233 | 1.363 ± 0.252 | 0.761 ± 0.172 | 1.220 ± 0.242 | 1.259 ± 0.442 | 1.963 ± 0.603 |
| Lead | 0.529 ± 0.557 | 6.754 ± 3.122 | 1.786 ± 0.748 | 3.661 ± 1.356 | 0.210 ± 0.424 | 4.466 ± 1.738 | 0.348 ± 0.335 | 2.903 ± 0.733 | 0.983 ± 0.904 | 2.186 ± 1.298 | 0.218 ± 0.352 | 3.053 ± 1.787 |
| Zinc | 269.918 ± 32.280 | 245.498 ± 28.891 | 289.965 ± 35.277 | 273.179 ± 34.400 | 267.931 ± 27.801 | 273.579 ± 31.399 | 273.100 ± 32.573 | 246.099 ± 18.666 | 264.488 ± 37.589 | 274.281 ± 41.358 | 271.527 ± 36.535 | 257.168 ± 24.199 |

*Sup. Inf. 8: Bioaccumulation factors of all analyzed metals in ryegrass, locusts and snails (taking into account soil concentration). The "P" and "P+S" accesses mean that animals had access to plants only or to plants and soil, respectively.*

| Species | Condition | Control | | Dis | | AgNPs | | AD-Control | | AD-Dis | | AD-AgNPs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Access | P | P+S | P | P+S | P | P+S | P | P+S | P | P+S | P | P+S |
| Ryegrass | Ag | 0.000 | | 0.000 | | *0.061 | | 0.000 | | 0.001 | | 0.003 | |
| | As | 0.214 | | 0.210 | | 0.239 | | 0.217 | | 0.228 | | 0.229 | |
| | Cd | 0.216 | | 0.188 | | *0.060 | | 0.186 | | 0.230 | | 0.165 | |
| | Cr | 0.017 | | 0.013 | | 0.009 | | 0.002 | | 0.002 | | 0.003 | |
| | Co | 0.019 | | 0.033 | | 0.029 | | 0.024 | | 0.033 | | 0.033 | |
| | Cu | 0.512 | | 0.541 | | *0.240 | | 0.406 | | 0.552 | | 0.523 | |
| | Mn | 0.161 | | 0.153 | | 0.147 | | 0.055 | | 0.052 | | 0.060 | |
| | Ni | 0.091 | | 0.091 | | 0.079 | | 0.049 | | 0.051 | | 0.059 | |
| | Pb | 0.000 | | 0.000 | | 0.000 | | 0.000 | | 0.000 | | 0.000 | |
| | Zn | 1.397 | | 1.029 | | 0.936 | | 0.847 | | 0.713 | | 0.780 | |
| Locust | Ag | 0.151 | 0.153 | 0.000 | 0.000 | 0.004 | 0.022 | 0.025 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | As | 0.206 | 0.239 | 0.189 | 0.213 | 0.154 | 0.131 | 0.225 | 0.194 | 0.201 | 0.173 | 0.144 | 0.116 |
| | Cd | 0.017 | 0.048 | 0.115 | 0.051 | 0.028 | 0.127 | 0.100 | 0.102 | 0.028 | 0.081 | 0.072 | 0.059 |
| | Cr | 0.137 | 0.066 | 0.180 | 0.323 | 0.360 | 0.451 | 0.128 | 0.283 | 0.282 | 0.432 | 0.269 | 0.489 |
| | Co | 0.024 | 0.021 | 0.025 | 0.044 | 0.068 | 0.083 | 0.014 | 0.053 | 0.069 | 0.057 | 0.052 | 0.045 |
| | Cu | 2.510 | 2.533 | 2.475 | 2.695 | 2.564 | 2.694 | 2.326 | 2.298 | 2.418 | 2.371 | 2.503 | 2.600 |
| | Mn | 0.012 | 0.013 | 0.012 | 0.013 | 0.013 | 0.023 | 0.007 | 0.011 | 0.008 | 0.013 | 0.010 | 0.016 |
| | Ni | 0.211 | 0.125 | 0.205 | 0.299 | 0.381 | 0.461 | 0.190 | 0.310 | 0.266 | 0.409 | 0.303 | 0.551 |
| | Pb | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | Zn | 3.253 | 3.243 | 3.214 | 3.469 | 3.161 | 3.294 | 2.090 | 2.133 | 2.088 | 2.170 | 2.304 | 2.421 |
| Snail | Ag | 0.004 | 0.042 | 0.132 | 0.249 | 0.025 | *1.367 | 0.057 | 0.075 | 0.041 | 0.030 | 0.015 | 0.329 |
| | As | 0.130 | 0.183 | 0.186 | 0.232 | 0.179 | 0.221 | 0.182 | 0.143 | 0.196 | 0.160 | 0.216 | 0.219 |
| | Cd | 3.448 | 6.340 | 3.703 | 6.793 | 2.936 | 6.049 | 3.428 | 5.125 | 3.373 | 5.224 | 3.098 | 6.417 |
| | Cr | 0.218 | 0.170 | 0.049 | 0.048 | 0.068 | 0.141 | 0.054 | 0.066 | 0.035 | 0.059 | 0.071 | 0.110 |
| | Co | 0.189 | 0.309 | 0.199 | 0.280 | 0.185 | 0.299 | 0.158 | 0.205 | 0.115 | 0.180 | 0.134 | 0.220 |
| | Cu | 8.314 | 8.602 | 8.508 | 9.515 | 9.426 | 9.420 | 7.284 | 6.870 | 6.638 | 8.056 | 8.331 | 8.818 |
| | Mn | 0.271 | 0.377 | 0.316 | 0.362 | 0.314 | 0.517 | 0.264 | 0.262 | 0.249 | 0.259 | 0.297 | 0.294 |
| | Ni | 0.263 | 0.234 | 0.102 | 0.092 | 0.112 | 0.183 | 0.094 | 0.115 | 0.064 | 0.103 | 0.113 | 0.176 |
| | Pb | 0.010 | 0.128 | 0.034 | 0.071 | 0.004 | 0.087 | 0.006 | 0.047 | 0.015 | 0.034 | 0.004 | 0.061 |
| | Zn | 5.423 | 4.932 | 5.915 | 5.572 | 5.677 | 5.796 | 3.577 | 3.223 | 3.667 | 3.803 | 3.929 | 3.722 |

## References

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# Chapitre V

# CONCLUSIONS et PERSPECTIVES

## 1) Principales conclusions

Les différentes expérimentations réalisées durant ce travail de thèse permettent d'éclaircir certains points. La digestion anaérobie des boues d'épuration, mimant un des processus de traitement des boues dans les stations d'épuration, entraîne la transformation chimique des AgNPs qui se sulfurent totalement, comme déjà observé précédemment dans la littérature. Après épandage, l'Ag sulfuré apporté par la boue dans un sol agricole est stable un certain temps et reste sulfuré au moins 5 semaines, confirmant la stabilité accrue des $Ag_2S$ comparés aux AgNPs. L'$Ag_2S$, à des concentrations réalistes (avoisinant les 10 mg $kg^{-1}$ de sol sec), n'affecte pas la biomasse et la survie des invertébrés étudiés ici : le vers de terre *Eisenia fetida* vivant en permanence dans le sol et le consommant, l'escargot *Cantareus aspersus* vivant à la surface du sol et pouvant consommer du sol pour compléter son alimentation végétale et le criquet *Locusta migratoria* vivant sur le couvert végétal. L'$Ag_2S$, apporté à des concentrations réalistes dans le sol, n'affecte pas la germination et la biomasse végétale de l'herbacée étudiée ici : le Ray gras anglais. De même, l'$Ag_2S$, à des concentrations réalistes, est nettement moins biodisponible pour les végétaux et animaux que les AgNPs ou ions $Ag^+$, bien que la bioaccumulation soit tout de même visible voire parfois significative. Le transfert significatif se fait préférentiellement vers les végétaux (de manière espèce-dépendante) ainsi que les animaux vivant en contact étroit avec le sol contaminé (vers de terre et escargots). Enfin, l'$Ag_2S$, apporté à des concentrations réalistes dans le sol, peut affecter de manière transitoire au moins les communautés actives de microorganismes du sol. Dans un sol plus sensible aux perturbations, cela peut entraîner la déstabilisation du cycle de l'azote, ce qui pourrait peut-être, dans un contexte d'expositions répétées, conduire à une altération de la fertilité des sols.

Ces expériences nous ont aussi permis de confirmer l'existence d'un mécanisme de régulation efficace de l'Ag chez le vers de terre *E. fetida*, et de nombreux indices indiquent que les protéines métallothionéines y prennent part, bien que l'expression du gène codant la CdMT ne montre pas systématiquement une régulation différentielle dans des conditions d'exposition à l'Ag.

Ainsi, bien que l'argent sulfuré soit nettement moins toxique et biodisponible que les AgNPs initiaux dont il est issu, des conséquences liées à l'accumulation de l'Ag dans les chaînes trophiques ne peuvent être exclues. En effet, cet Ag dispersé dans les sols pourrait s'accumuler dans les végétaux et les invertébrés du sol et ainsi être transmis à d'autres niveaux trophiques, y compris l'homme puisque les teneurs en Ag dans les denrées alimentaires ne sont pas surveillées. En revanche, on ne peut exclure des conséquences sur le bon fonctionnement des sols, et la continuité des services écosystémiques. En effet, une altération des cycles de nutriments, tels que les nutriments azotés, pourrait avoir un impact sur la fertilité des sols à long terme et ainsi réduire les services d'approvisionnement rendus par les sols, en affectant l'économie agricole.

## 2) Travaux futurs et perspectives

Afin d'approfondir ces résultats et de comprendre les mécanismes de toxicité et/ou de détoxication de l'Ag chez les organismes, il serait très intéressant de réaliser une étude transcriptomique chez des organismes modèles animaux et végétaux (comme *E. fetida* et *Brassica napus*). Ainsi, en comparant ces organismes en condition contrôle et en condition exposée à l'$Ag_2S$, cela permettrait d'observer les gènes qui sont différentiellement transcrits, et ainsi comprendre quels mécanismes jouent un rôle en cas d'exposition à l'Ag.

En plus d'études plutôt fonctionnelles, il serait important de poursuivre les études écotoxicologiques et environnementales afin de pouvoir conclure plus sûrement sur l'écotoxicité et les risques associés aux dérivés des AgNPs dans l'environnement terrestre.

Des études de terrain sur l'accumulation de l'Ag dans les sols et le transfert de l'Ag vers les végétaux cultivés ainsi que divers invertébrés et autres animaux de l'écosystème terrestre permettraient d'obtenir une vision plus globale et réaliste du problème de transfert trophique potentiel. De même, des études à très long terme (quelques années) sur l'impact des boues contaminées par l'Ag sur la biodiversité et les activités principales des microorganismes du sol permettraient d'évaluer l'impact réel de cette pollution sur le potentiel fertile des sols et l'impact éventuel sur les services écosystémiques rendus par les microorganismes.

Il serait également important de prendre en compte et mesurer l'effet cocktail potentiel. En effet, les boues d'épuration sont riches en divers contaminants et notamment en d'autres métaux très toxiques comme le Cd. L'étude des effets de l'Ag s'est concentrée et se concentre encore sur l'étude de boues contaminées par de l'Ag, mais très peu contaminée en d'autres éléments. Ces études sont indispensables pour comprendre l'effet de l'Ag dans l'environnement, mais en réalité, malgré des contrôles systématiques pour certains polluants, en épandant les boues d'épuration, on applique sur les sols une multitude de contaminants, métalliques, organiques, plastiques… qui ensemble peuvent impacter différemment les organismes vivants. Ainsi des études à long terme in situ sur l'épandage de ces boues multi-contaminées semblent indispensables pour avoir une vision globale des risques associés à l'épandage des boues d'épuration.

De plus, la digestion anaérobie est une méthode de traitement des boues d'épuration par stabilisation biologique. Or, il en existe bien d'autres comme la stabilisation biologique aérobie, la stabilisation chimique (chaulage par exemple) ou encore la déshydratation totale par séchage thermique. Puisque la sulfuration des AgNPs commence dès la circulation des AgNPs dans les

canalisations d'eaux usées et durant le traitement des eaux, il est possible qu'une part considérable de cet Ag se retrouve également sulfuré. Cependant, d'autres transformations pourraient éventuellement avoir lieu. Il serait ainsi intéressant de contrôler la spéciation de l'Ag dans ces différentes boues ayant subi divers procédés de stabilisation et hygiénisation.

En attendant de plus amples résultats quant aux risques encourus par le rejet de ces dérivés d'AgNPs dans les sols, il serait utile de doser l'Ag dans les boues d'épuration, en même temps que les autres métaux déjà surveillés, afin de pouvoir réaliser un choix raisonné sur le devenir des boues les plus concentrées en Ag. En effet, en fonction de l'état des sols (plus ou moins contaminés) et de leur usage (type de culture), l'ajout de boues contaminées n'aura pas le même impact – le pire des scenarios étant l'épandage d'une boue fortement multi-contaminée sur un sol peu pollué, où la communauté de microorganismes pourrait être très sensible à de tels apports, et où les cultures correspondent à des espèces végétales qui accumulent aisément les contaminants dans leurs parties comestibles. De plus, les boues d'épuration sont recyclées de diverses façons. L'épandage représente une part considérable de la valorisation de ce déchet, mais l'incinération avec valorisation énergétique, l'enfouissement ou encore le compostage sont aussi fréquemment utilisés. La remédiation de sols fortement pollués par les boues est également une pratique envisageable. Le contrôle systématique des teneurs en Ag, tout comme d'autres contaminants émergeants qui ne sont toujours pas contrôlés, permettrait ainsi de rediriger les boues vers des filières d'élimination différentes afin de limiter les conséquences sur l'environnement et l'économie agricole.

# REFERENCES

Adam, V., Caballero-Guzman, A., Nowack, B., 2018. Considering the forms of released engineered nanomaterials in probabilistic material flow analysis. Environ. Pollut. 243, 17–27. https://doi.org/10.1016/j.envpol.2018.07.108

Anderson, J.P.E., Domsch, K.H., 1978. A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem. 10, 215–221. https://doi.org/10.1016/0038-0717(78)90099-8

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance 26, 32–46.

ANSES, 2015. Avis de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail relatif à l'expertise concernant la mise à jour des connaissances sur « l'évaluation des risques sanitaires et environnementaux liés à l'exposition aux nanoparticules d'argent ». URL https://www.anses.fr/fr/system/files/AP2011sa0224Ra.pdf.

Asadishad, B., Chahal, S., Akbari, A., Cianciarelli, V., Azodi, M., Ghoshal, S., Tufenkji, N., 2018. Amendment of Agricultural Soil with Metal Nanoparticles: Effects on Soil Enzyme Activity and Microbial Community Composition. Environ. Sci. Technol. 52, 1908–1918. https://doi.org/10.1021/acs.est.7b05389

Baalousha, M., Arkill, K.P., Romer, I., Palmer, R.E., Lead, J.R., 2015. Transformations of citrate and Tween coated silver nanoparticles reacted with $Na_2S$. Sci. Total Environ. 502, 344–353. https://doi.org/10.1016/j.scitotenv.2014.09.035

Baccaro, M., Harrison, S., van den Berg, H., Sloot, L., Hermans, D., Cornelis, G., van Gestel, C.A.M., van den Brink, N.W., 2019. Bioturbation of $Ag_2S$-NPs in soil columns by earthworms. Environ. Pollut. 252, 155–162. https://doi.org/10.1016/j.envpol.2019.05.106

Baccaro, M., Undas, A.K., de Vriendt, J., van den Berg, J.H.J., Peters, R.J.B., van den Brink, N.W., 2018. Ageing, dissolution and biogenic formation of nanoparticles: how do these factors affect the uptake kinetics of silver nanoparticles in earthworms? Environ. Sci. Nano 5, 1107–1116. https://doi.org/10.1039/C7EN01212H

Barbasz, A., Kreczmer, B., Oćwieja, M., 2016. Effects of exposure of callus cells of two wheat varieties to silver nanoparticles and silver salt (AgNO3). Acta Physiol. Plant. 38, 76. https://doi.org/10.1007/s11738-016-2092-z

Barker, G., Watts, C., 2002. Management of the invasive alien snail Cantareus aspersus on conservation land.

Barker, L.K., Giska, J.R., Radniecki, T.S., Semprini, L., 2018. Effects of short- and long-term exposure of silver nanoparticles and silver ions to Nitrosomonas europaea biofilms and planktonic cells. Chemosphere 206, 606–614. https://doi.org/10.1016/j.chemosphere.2018.05.017

Baroudi, F., Al Alam, J., Fajloun, Z., Millet, M., 2020. Snail as sentinel organism for monitoring the environmental pollution; a review. Ecol. Indic. 113, 106240. https://doi.org/10.1016/j.ecolind.2020.106240

Barrena, R., Casals, E., Colón, J., Font, X., Sánchez, A., Puntes, V., 2009. Evaluation of the ecotoxicity of model nanoparticles. Chemosphere 75, 850–857. https://doi.org/10.1016/j.chemosphere.2009.01.078

Beddow, J., Stolpe, B., Cole, P., Lead, J.R., Sapp, M., Lyons, B.P., Colbeck, I., Whitby, C., 2014. Effects of engineered silver nanoparticles on the growth and activity of ecologically important microbes: Impact of silver nanoparticles on environmental microbes. Environ. Microbiol. Rep. 6, 448–458. https://doi.org/10.1111/1758-2229.12147

Beeby, A., 1985. The Role of Helix aspersa as a Major Herbivore in the Transfer of Lead Through a Polluted Ecosystem. J. Appl. Ecol. 22, 267–275. https://doi.org/10.2307/2403344

Bernard, F., Brulle, F., Douay, F., Lemière, S., Demuynck, S., Vandenbulcke, F., 2010. Metallic trace element body burdens and gene expression analysis of biomarker candidates in *Eisenia fetida*, using an "exposure/depuration" experimental scheme with field soils. Ecotoxicol. Environ. Saf. 73, 1034–1045. https://doi.org/10.1016/j.ecoenv.2010.01.010

Bernard, F., Brulle, F., Dumez, S., Lemiere, S., Platel, A., Nesslany, F., Cuny, D., Deram, A., Vandenbulcke, F., 2015. Antioxidant responses of Annelids, Brassicaceae and Fabaceae to pollutants: A review. Ecotoxicol. Environ. Saf. 114, 273–303. https://doi.org/10.1016/j.ecoenv.2014.04.024

Bicho, R.C., Ribeiro, T., Rodrigues, N.P., Scott-Fordsmand, J.J., Amorim, M.J.B., 2016. Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*. J. Hazard. Mater. 318, 608–614. https://doi.org/10.1016/j.jhazmat.2016.07.040

Blais, J. F., Tyagi, R. D., Auclair, J. C., 1993. Bioleaching of metals from sewage sludge: Microorganisms and growth kinetics. Water Res. 27, 101–110. https://doi.org/10.1016/0043-1354(93)90200-2

Blaser, S.A., Scheringer, M., MacLeod, M., Hungerbühler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano-functionalized plastics and textiles. Sci. Total Environ. 390, 396–409. https://doi.org/10.1016/j.scitotenv.2007.10.010

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 57–59. https://doi.org/10.1038/nmeth.2276

Bone, A.J., Colman, B.P., Gondikas, A.P., Newton, K.M., Harrold, K.H., Cory, R.M., Unrine, J.M., Klaine, S.J., Matson, C.W., Di Giulio, R.T., 2012. Biotic and Abiotic Interactions in Aquatic Microcosms Determine Fate and Toxicity of Ag Nanoparticles: Part 2–Toxicity and Ag Speciation. Environ. Sci. Technol. 46, 6925–6933. https://doi.org/10.1021/es204683m

Bourdineaud, J.-P., Štambuk, A., Šrut, M., Radić Brkanac, S., Ivanković, D., Lisjak, D., Sauerborn Klobučar, R., Dragun, Z., Bačić, N., Klobučar, G.I.V., 2019. Gold and silver nanoparticles effects to the earthworm *Eisenia fetida* – the importance of tissue over soil concentrations. Drug Chem. Toxicol. 1–18. https://doi.org/10.1080/01480545.2019.1567757

Brami, C., Glover, A.R., Butt, K.R., Lowe, C.N., 2017. Effects of silver nanoparticles on survival, biomass change and avoidance behaviour of the endogeic earthworm *Allolobophora chlorotica*. Ecotoxicol. Environ. Saf. 141, 64–69. https://doi.org/10.1016/j.ecoenv.2017.03.015

Brulle, F., Cocquerelle, C., Wamalah, A.N., Morgan, A.J., Kille, P., Leprêtre, A., Vandenbulcke, F., 2008. cDNA cloning and expression analysis of *Eisenia fetida* (Annelida: Oligochaeta) phytochelatin synthase under cadmium exposure. Ecotoxicol. Environ. Saf. 71, 47–55. https://doi.org/10.1016/j.ecoenv.2007.10.032

Brulle, F., Lemière, S., Waterlot, C., Douay, F., Vandenbulcke, F., 2011. Gene expression analysis of 4 biomarker candidates in *Eisenia fetida* exposed to an environmental metallic trace elements gradient: A microcosm study. Sci. Total Environ. 409, 5470–5482. https://doi.org/10.1016/j.scitotenv.2011.08.040

Brulle, F., Mitta, G., Cocquerelle, C., Vieau, D., Lemière, S., Leprêtre, A., Vandenbulcke, F., 2006. Cloning and Real-Time PCR Testing of 14 Potential Biomarkers in *Eisenia fetida* Following Cadmium Exposure. Environ. Sci. Technol. 40, 2844–2850. https://doi.org/10.1021/es052299x

Burford, J.R., Bremner, J.M., 1975. Relationships between the denitrification capacities of soils and total, water-soluble and readily decomposable soil organic matter. Soil Biol. Biochem. 7, 389–394. https://doi.org/doi.org/10.1016/0038-0717(75)90055-3

Calleja-Cervantes, M.E., Aparicio-Tejo, P.M., Villadas, P.J., Irigoyen, I., Irañeta, J., Fernández-González, A.J., Fernández-López, M., Menéndez, S., 2017. Rational application of treated sewage sludge with urea increases GHG mitigation opportunities in Mediterranean soils. Agric. Ecosyst. Environ., Quantification and mitigation of greenhouse gas emissions in Mediterranean cropping systems 238, 114–127. https://doi.org/10.1016/j.agee.2016.09.021

Capek, I., 2004. Preparation of metal nanoparticles in water-in-oil (w/o) microemulsions. Adv. Colloid Interface Sci. 110, 49–74. https://doi.org/10.1016/j.cis.2004.02.003

Carbone, D., Faggio, C., 2019. Helix aspersa as sentinel of development damage for biomonitoring purpose: A validation study. Mol. Reprod. Dev. 86, 1283–1291. https://doi.org/10.1002/mrd.23117

Carbonell, G., Pro, J., Gómez, N., Babín, M.M., Fernández, C., Alonso, E., Tarazona, J.V., 2009. Sewage sludge applied to agricultural soil: Ecotoxicological effects on representative soil organisms. Ecotoxicol. Environ. Saf. 72, 1309–1319. https://doi.org/10.1016/j.ecoenv.2009.01.007

Castellano, J.J., Shafii, S.M., Ko, F., Donate, G., Wright, T.E., Mannari, R.J., Payne, W.G., Smith, D.J., Robson, M.C., 2007. Comparative evaluation of silver-containing antimicrobial dressings and drugs. Int. Wound J. 4, 114–122. https://doi.org/10.1111/j.1742-481X.2007.00316.x

Chaurand, P., Liu, W., Borschneck, D., Levard, C., Auffan, M., Paul, E., Collin, B., Kieffer, I., Lanone, S., Rose, J., Perrin, J., 2018. Multi-scale X-ray computed tomography to detect and localize metal-based nanomaterials in lung tissues of in vivo exposed mice. Sci. Rep. 8, 1–11. https://doi.org/10.1038/s41598-018-21862-4

Chen, C., Zhou, Q., Liu, S., Xiu, Z., 2011. Acute toxicity, biochemical and gene expression responses of the earthworm *Eisenia fetida* exposed to polycyclic musks. Chemosphere 83, 1147–1154. https://doi.org/10.1016/j.chemosphere.2011.01.006

Chen, X., Schluesener, H.J., 2008. Nanosilver: A nanoproduct in medical application. Toxicol. Lett. 176, 1–12. https://doi.org/10.1016/j.toxlet.2007.10.004

Chen, Y., Mao, Y., Song, M., Yin, Y., Liu, G., Cai, Y., 2020. Occurrence and leaching of silver in municipal sewage sludge in China. Ecotoxicol. Environ. Saf. 189. https://doi.org/10.1016/j.ecoenv.2019.109929

Chhipa, H., 2019. Applications of nanotechnology in agriculture. Methods Microbiol. 115–142.

Choi, J.-M., Han, S.-K., Lee, C.-Y., 2018. Enhancement of methane production in anaerobic digestion of sewage sludge by thermal hydrolysis pretreatment. Bioresour. Technol. 259, 207–213. https://doi.org/10.1016/j.biortech.2018.02.123

Choi, J.S., Park, J.-W., 2015. Molecular characterization and toxicological effects of citrate-coated silver nanoparticles in a terrestrial invertebrate, the earthworm (*Eisenia fetida*). Mol. Cell. Toxicol. 11, 423–431. https://doi.org/10.1007/s13273-015-0045-z

Choi, O., Deng, K.K., Kim, N.-J., Ross, L., Surampalli, R.Y., Hu, Z., 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. Water Res. 42, 3066–3074. https://doi.org/10.1016/j.watres.2008.02.021

Choi, O., Hu, Z., 2008. Size Dependent and Reactive Oxygen Species Related Nanosilver Toxicity to Nitrifying Bacteria. Environ. Sci. Technol. 42, 4583–4588. https://doi.org/10.1021/es703238h

Circular DE / GE n ° 357 of 03/16/99, 1999. Circular DE / GE n ° 357 of 03/16/99 relating to the regulations relating to the spreading of sludge from urban wastewater treatment plants.

Coeurdassier, M., Vaufleury, A.G., Lovy, C., Badot, P.-M., 2002. Is the cadmium uptake from soil important in bioaccumulation and toxic effects for snails? Ecotoxicol. Environ. Saf. 53, 425–431. https://doi.org/10.1016/S0147-6513(02)00004-0

Collin, B., Tsyusko, O.V., Starnes, D.L., Unrine, J.M., 2016. Effect of natural organic matter on dissolution and toxicity of sulfidized silver nanoparticles to *Caenorhabditis elegans*. Environ. Sci. Nano 3, 728–736. https://doi.org/10.1039/C6EN00095A

Colman, B.P., Arnaout, C.L., Anciaux, S., Gunsch, C.K., Hochella, M.F., Kim, B., Lowry, G.V., McGill, B.M., Reinsch, B.C., Richardson, C.J., Unrine, J.M., Wright, J.P., Yin, L., Bernhardt, E.S., 2013. Low Concentrations of Silver Nanoparticles in Biosolids Cause Adverse Ecosystem Responses under Realistic Field Scenario. PLoS ONE 8, e57189. https://doi.org/10.1371/journal.pone.0057189

Cornelis, G., Thomas, C.D., McLaughlin, M.J., Kirby, J.K., Beak, D.G., Chittleborough, D., 2012. Retention and Dissolution of Engineered Silver Nanoparticles in Natural Soils. Soil Sci. Soc. Am. J. 76, 891–902. https://doi.org/10.2136/sssaj2011.0360

Courtois, P., Rorat, A., Lemiere, S., Guyoneaud, R., Attard, E., Levard, C., Vandenbulcke, F., 2019. Ecotoxicology of silver nanoparticles and their derivatives introduced in soil with or without sewage sludge: A review of effects on microorganisms, plants and animals. Environ. Pollut. 253, 578–598. https://doi.org/10.1016/j.envpol.2019.07.053

Courtois, P., Rorat, A., Lemiere, S., Levard, C., Chaurand, P., Grobelak, A., Lors, C., Vandenbulcke, F., 2020a. Accumulation, speciation and localization of silver nanoparticles in the earthworm *Eisenia fetida*. Environ. Sci. Pollut. Res. Int. https://doi.org/10.1007/s11356-020-08548-z

Courtois, P., Rorat, A., Lemiere, S., Guyoneaud, R., Attard, E., Longepierre, M., Rigal, F., Levard, C., Chaurand, P., Grosser, A., Grobelak, A., Kacprzak, M., Lors, C., Richaume, A., Vandenbulcke, F., 2020b. Medium-Term Effects of Ag Supplied Directly or via Sewage Sludge to an Agricultural Soil on *Eisenia Fetida* Earthworm and Soil Microbial Communities. Chemosphere, 128761. https://doi.org/10.1016/j.chemosphere.2020.128761.

Coutris, C., Hertel-Aas, T., Lapied, E., Joner, E.J., Oughton, D.H., 2011. Bioavailability of cobalt and silver nanoparticles to the earthworm *Eisenia fetida*. Nanotoxicology 6, 186–195. https://doi.org/10.3109/17435390.2011.569094

Curieses Silvana, P., García-Velasco, N., Urionabarrenetxea, E., Elena, S.M., Bilbao, E., Di Marzio Walter, D., Soto, M., 2017. Responses to silver nanoparticles and silver nitrate in a battery of biomarkers measured in coelomocytes and in target tissues of *Eisenia fetida* earthworms. Ecotoxicol. Environ. Saf. 141, 57–63. https://doi.org/10.1016/j.ecoenv.2017.03.008

Cvjetko, P., Milošić, A., Domijan, A.-M., Vinković Vrček, I., Tolić, S., Peharec Štefanić, P., Letofsky-Papst, I., Tkalec, M., Balen, B., 2017. Toxicity of silver ions and differently coated silver nanoparticles in *Allium cepa* roots. Ecotoxicol. Environ. Saf. 137, 18–28. https://doi.org/10.1016/j.ecoenv.2016.11.009

Dallinger, R., 1996. Metallothionein research in terrestrial invertebrates: Synopsis and perspectives. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 113, 125–133. https://doi.org/10.1016/0742-8413(95)02078-0

Dang, F., Chen, Y.-Z., Huang, Y.-N., Hintelmann, H., Si, Y.-B., Zhou, D.-M., 2019. Discerning the Sources of Silver Nanoparticle in a Terrestrial Food Chain by Stable Isotope Tracer Technique. Environ. Sci. Technol. 53, 3802–3810. https://doi.org/10.1021/acs.est.8b06135

Das, P., Barua, S., Sarkar, S., Chatterjee, S.K., Mukherjee, S., Goswami, L., Das, S., Bhattacharya, S., Karak, N., Bhattacharya, S.S., 2018. Mechanism of toxicity and transformation of silver nanoparticles: Inclusive assessment in earthworm-microbe-soil-plant system. Geoderma 314, 73–84. https://doi.org/10.1016/j.geoderma.2017.11.008

Davidson, S.K., Powell, R., James, S., 2013. A global survey of the bacteria within earthworm nephridia. Mol. Phylogenet. Evol. 67, 188–200. https://doi.org/10.1016/j.ympev.2012.12.005

De Cáceres, M., Legendre, P., Moretti, M., 2010. Improving indicator species analysis by combining groups of sites. Oikos 119, 1674–1684. https://doi.org/10.1111/j.1600-0706.2010.18334.x

De Vaufleury, A., 2015. Landsnail for Ecotoxicological Assessment of Chemicals and Soil Contamination – Ecotoxicological Assessment of Chemicals and Contaminated Soils Using the Terrestrial Snail, *Helix aspersa*, at Various Stage of Its Life Cycle: A Review, in: Armon, R.H., Hänninen, O. (Eds.), Environmental Indicators. Springer Netherlands, Dordrecht, pp. 365–391. https://doi.org/10.1007/978-94-017-9499-2_23

Demuynck, S., Grumiaux, F., Mottier, V., Schikorski, D., Lemière, S., Leprêtre, A., 2006. Metallothionein response following cadmium exposure in the oligochaete *Eisenia fetida*. Comp. Biochem. Physiol. Toxicol. Pharmacol. CBP 144, 34–46. https://doi.org/10.1016/j.cbpc.2006.05.004

Devkota, B., Schmidt, G.H., 2000. Accumulation of heavy metals in food plants and grasshoppers from the Taigetos Mountains, Greece. Agric. Ecosyst. Environ. 78, 85–91. https://doi.org/10.1016/S0167-8809(99)00110-3

Diez-Ortiz, M., Lahive, E., George, S., Ter Schure, A., Van Gestel, C.A.M., Jurkschat, K., Svendsen, C., Spurgeon, D.J., 2015a. Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; bioavailability and toxicity of silver ions ($AgNO_3$) and silver nanoparticles to earthworm *Eisenia fetida* in long-term aged soils. Environ. Pollut. 203, 191–198. https://doi.org/10.1016/j.envpol.2015.03.033

Diez-Ortiz, M., Lahive, E., Kille, P., Powell, K., Morgan, A.J., Jurkschat, K., Van Gestel, C.A.M., Mosselmans, J.F.W., Svendsen, C., Spurgeon, D.J., 2015b. Uptake routes and toxicokinetics of silver nanoparticles and silver ions in the earthworm *Lumbricus rubellus*. Environ. Toxicol. Chem. 34, 2263–2270. https://doi.org/10.1002/etc.3036

Diogène, J., Dufour, M., Poirier, G.G., Nadeau, D., 1997. Extrusion of earthworm coelomocytes: comparison of the cell populations recovered from the species *Lumbricus terrestris*, *Eisenia fetida* and *Octolasion tyrtaeum*. Lab. Anim. 31, 326–336. https://doi.org/10.1258/002367797780596068

Directive 86/278/ECC, 1986. Directive n° 86/278 du 12/06/86 relative à la protection de l'environnement et notamment des sols, lors de l'utilisation des boues d'épuration en agriculture | AIDA. URL https://aida.ineris.fr/consultation_document/1081.

Directive 2002/32 of European Commission, 2002. Directive 2002/32/CE du Parlement Européen et du Conseil du 7 mai 2002 sur les substances indésirables dans les aliments pour animaux. ResearchGate.

Directive européenne 86/278/CEE, 1986. Directive du Conseil du 12 juin 1986 relative à la protection de l'environnement et notamment des sols, lors de l'utilisation des boues d'épuration en agriculture (86/278/CEE).

Domingos, R.F., Baalousha, M.A., Ju-Nam, Y., Reid, M.M., Tufenkji, N., Lead, J.R., Leppard, G.G., Wilkinson, K.J., 2009. Characterizing Manufactured Nanoparticles in the Environment: Multimethod Determination of Particle Sizes. Environ. Sci. Technol. 43, 7277–7284. https://doi.org/10.1021/es900249m

Donner, E., Scheckel, K., Sekine, R., Popelka-Filcoff, R.S., Bennett, J.W., Brunetti, G., Naidu, R., McGrath, S.P., Lombi, E., 2015. Non-labile silver species in biosolids remain stable throughout 50 years of weathering and ageing. Environ. Pollut. 205, 78–86. https://doi.org/10.1016/j.envpol.2015.05.017

Doolette, C.L., Gupta, V.V.S.R., Lu, Y., Payne, J.L., Batstone, D.J., Kirby, J.K., Navarro, D.A., McLaughlin, M.J., 2016. Quantifying the Sensitivity of Soil Microbial Communities to Silver Sulfide Nanoparticles Using Metagenome Sequencing. PLOS ONE 11, e0161979. https://doi.org/10.1371/journal.pone.0161979

Doolette, C.L., McLaughlin, M.J., Kirby, J.K., Navarro, D.A., 2015. Bioavailability of silver and silver sulfide nanoparticles to lettuce (*Lactuca sativa*): Effect of agricultural amendments on plant uptake. J. Hazard. Mater. 300, 788–795. https://doi.org/10.1016/j.jhazmat.2015.08.012

DRAAF, N.-P. de C., 2013. Inventaire, Gestion et Conservation des Sols dans le Nord - Pas de Clais. URL http://draaf.hauts-de-france.agriculture.gouv.fr/IMG/pdf/Demarche_IGCS_dans_le_Nord-Pas_de_Calais_v2013-04-06_cle014cb8.pdf.

Dufrene, M., Legendre, P., 1997. Species Assemblages and Indicator Species: The Need for a Flexible Asymmetrical Approach. Ecol. Monogr. 67, 345–366. https://doi.org/10.2307/2963459

Durenkamp, M., Pawlett, M., Ritz, K., Harris, J.A., Neal, A.L., McGrath, S.P., 2016. Nanoparticles within WWTP sludges have minimal impact on leachate quality and soil microbial community structure and function. Environ. Pollut. 211, 399–405. https://doi.org/10.1016/j.envpol.2015.12.063

El-Temsah, Y.S., Joner, E.J., 2012. Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil. Environ. Toxicol. 27, 42–49. https://doi.org/10.1002/tox.20610

Enwall, K., Philippot, L., Hallin, S., 2005. Activity and Composition of the Denitrifying Bacterial Community Respond Differently to Long-Term Fertilization. Appl. Environ. Microbiol. 71, 8335–8343. https://doi.org/10.1128/AEM.71.12.8335-8343.2005

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., Maman, S., Hernandez-Raquet, G., Combes, S., Pascal, G., 2018. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Bioinforma. Oxf. Engl. 34, 1287–1294. https://doi.org/10.1093/bioinformatics/btx791

European commission, 2017. Report from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the committee of the regions on implementation of free trade agreements.

European commission, 2014. Nanosilver: Safety, health and environmental effects and role in antimicrobial resistance. Mater. Today 18, 122–123. https://doi.org/10.1016/j.mattod.2015.02.014

European commission, 2012. COMMISSION STAFF WORKING PAPER Types and uses of nanomaterials, including safety aspects Accompanying the Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee on the Second Regulatory Review on Nanomaterials, CELEX1.

Eurostat, 2020. Eurostat - Sewage sludge production and disposal (data for 2017). URL https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do.

Eurostat, 2017. Sewage sludge disposal from urban wastewater treatment, by treatment method, 2017 (% of total dry mass) - Statistics Explained.

Eyambe, G.S., Goven, A.J., Fitzpatrick, L.C., Venables, B.J., Cooper, E.L., 1991. A non-invasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies. Lab. Anim. 25, 61–67. https://doi.org/10.1258/002367791780808095

Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. Environ. Int. 37, 517–531. https://doi.org/10.1016/j.envint.2010.10.012

Fijalkowski, K., Rorat, A., Grobelak, A., Kacprzak, M.J., 2017. The presence of contaminations in sewage sludge – The current situation. J. Environ. Manage., Environmental management as a pillar for sustainable development 203, 1126–1136. https://doi.org/10.1016/j.jenvman.2017.05.068

Fischer, E., 1993. The myelo-erythroid nature of the chloragogenous-like tissues of the annelids. Comp. Biochem. Physiol. A Physiol. 106, 449–453. https://doi.org/10.1016/0300-9629(93)90237-X

Food and Agriculture Organization of the United Nations, 2015. World reference base for soil resources 2014: international soil classification system for naming soils and creating legends for soil maps. FAO, Rome.

Fudou, R., Jojima, Y., Iizuka, T., Yamanaka, S., 2002. *Haliangium ochraceum gen. nov., sp. nov.* and *Haliangium tepidum sp.* nov.: Novel moderately halophilic myxobacteria isolated from coastal saline environments. J. Gen. Appl. Microbiol. 48, 109–115. https://doi.org/10.2323/jgam.48.109

Gagnon, V., Button, M., Boparai, H.K., Nearing, M., O'Carroll, D.M., Weber, K.P., 2019. Influence of realistic wearing on the morphology and release of silver nanomaterials from textiles. Environ. Sci. Nano 6, 411–424. https://doi.org/10.1039/C8EN00803E

Garcia-Velasco, N., Gandariasbeitia, M., Irizar, A., Soto, M., 2016. Uptake route and resulting toxicity of silver nanoparticles in *Eisenia fetida* earthworm exposed through Standard OECD Tests. Ecotoxicology 25, 1543–1555. https://doi.org/10.1007/s10646-016-1710-2

Garcia-Velasco, N., Peña-Cearra, A., Bilbao, E., Zaldibar, B., Soto, M., 2017. Integrative assessment of the effects produced by Ag nanoparticles at different levels of biological complexity in *Eisenia fetida* maintained in two standard soils (OECD and LUFA 2.3). Chemosphere 181, 747–758. https://doi.org/10.1016/j.chemosphere.2017.04.143

Geisler-Lee, J., Brooks, M., Gerfen, J., Wang, Q., Fotis, C., Sparer, A., Ma, X., Berg, R., Geisler, M., 2014. Reproductive Toxicity and Life History Study of Silver Nanoparticle Effect, Uptake and Transport in *Arabidopsis thaliana*. Nanomaterials 4, 301–318. https://doi.org/10.3390/nano4020301

Geisler-Lee, J., Wang, Q., Yao, Y., Zhang, W., Geisler, M., Li, K., Huang, Y., Chen, Y., Kolmakov, A., Ma, X., 2012. Phytotoxicity, accumulation and transport of silver nanoparticles by *Arabidopsis thaliana*. Nanotoxicology 7, 323–337. https://doi.org/10.3109/17435390.2012.658094

Gimbert, F., de Vaufleury, A., Douay, F., Scheifler, R., Coeurdassier, M., Badot, P.-M., 2006. Modelling chronic exposure to contaminated soil: A toxicokinetic approach with the terrestrial snail *Helix aspersa*. Environ. Int. 32, 866–875. https://doi.org/10.1016/j.envint.2006.05.006

Gomes, S.I.L., Hansen, D., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Effects of silver nanoparticles to soil invertebrates: Oxidative stress biomarkers in *Eisenia fetida*. Environ. Pollut. 199, 49–55. https://doi.org/10.1016/j.envpol.2015.01.012

Gomes, S.I.L., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2013. Mechanisms of response to silver nanoparticles on *Enchytraeus albidus* (Oligochaeta): Survival, reproduction and gene expression profile. J. Hazard. Mater. 254–255, 336–344. https://doi.org/10.1016/j.jhazmat.2013.04.005

Gomot, A., Gomot, L., Boukraa, S., Bruckert, S., 1989. Influence of soil on the growth of the land snail *Helix aspersa*. An experimental study of the absorption route for the stimulating factors. J. Molluscan Stud. 55, 1–7. https://doi.org/10.1093/mollus/55.1.1-a

Gomot de Vaufleury, A., 2000. Standardized Growth Toxicity Testing (Cu, Zn, Pb, and Pentachlorophenol) with *Helix aspersa*. Ecotoxicol. Environ. Saf. 46, 41–50. https://doi.org/10.1006/eesa.1999.1872

Gomot de Vaufleury, A., Pihan, F., 2002. Methods for toxicity assessment of contaminated soil by oral or dermal uptake in land snails: Metal bioavailability and bioaccumulation. Environ. Toxicol. Chem. 21, 820–827. https://doi.org/10.1002/etc.5620210419

Gottschalk, F., Sun, T., Nowack, B., 2013. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. Environ. Pollut. 181, 287–300. https://doi.org/10.1016/j.envpol.2013.06.003

Grosser, A., 2017. The influence of decreased hydraulic retention time on the performance and stability of co-digestion of sewage sludge with grease trap sludge and organic fraction of municipal waste. J. Environ. Manage., Environmental management as a pillar for sustainable development 203, 1143–1157. https://doi.org/10.1016/j.jenvman.2017.04.085

Grosser, A., Neczaj, E., 2018. Sewage sludge and fat rich materials co-digestion - Performance and energy potential. J. Clean. Prod. 198, 1076–1089. https://doi.org/10.1016/j.jclepro.2018.07.124

Grün, A.-L., Emmerling, C., 2018. Long-term effects of environmentally relevant concentrations of silver nanoparticles on major soil bacterial phyla of a loamy soil. Environ. Sci. Eur. 30, 31. https://doi.org/10.1186/s12302-018-0160-2

Grün, A.-L., Straskraba, S., Schulz, S., Schloter, M., Emmerling, C., 2018. Long-term effects of environmentally relevant concentrations of silver nanoparticles on microbial biomass, enzyme activity, and functional genes involved in the nitrogen cycle of loamy soil. J. Environ. Sci. 69, 12–22. https://doi.org/10.1016/j.jes.2018.04.013

Hamed, S.S., Kauschke, E., Cooper, E.L., 2002. Cytochemical Properties of Earthworm Coelomocytes Enriched by Percoll, in: A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications., NATO Science Series. https://doi.org/10.3923/ijzr.2005.74.83

Hammer, O., Harper, D.A.T., Ryan, P.D., 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis 9.

Hänsch, M., Emmerling, C., 2010. Effects of silver nanoparticles on the microbiota and enzyme activity in soil. J. Plant Nutr. Soil Sci. 173, 554–558. https://doi.org/10.1002/jpln.200900358

Hayashi, Y., Heckmann, L.-H., Simonsen, V., Scott-Fordsmand, J.J., 2013. Time-course profiling of molecular stress responses to silver nanoparticles in the earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. 98, 219–226. https://doi.org/10.1016/j.ecoenv.2013.08.017

Hayashi, Y., Miclaus, T., Engelmann, P., Autrup, H., Sutherland, D.S., Scott-Fordsmand, J.J., 2015. Nanosilver pathophysiology in earthworms: Transcriptional profiling of secretory proteins and the implication for the protein corona. Nanotoxicology 10, 303–311. https://doi.org/10.3109/17435390.2015.1054909

He, S., Feng, Youzhi, Ni, J., Sun, Y., Xue, L., Feng, Yanfang, Yu, Y., Lin, X., Yang, L., 2016. Different responses of soil microbial metabolic activity to silver and iron oxide nanoparticles. Chemosphere 147, 195–202. https://doi.org/10.1016/j.chemosphere.2015.12.055

Heckmann, L.-H., Hovgaard, M.B., Sutherland, D.S., Autrup, H., Besenbacher, F., Scott-Fordsmand, J.J., 2011. Limit-test toxicity screening of selected inorganic nanoparticles

to the earthworm *Eisenia fetida*. Ecotoxicology 20, 226–233. https://doi.org/10.1007/s10646-010-0574-0

Höckner, M., Dallinger, R., Stürzenbaum, S.R., 2011. Nematode and snail metallothioneins. JBIC J. Biol. Inorg. Chem. 16, 1057. https://doi.org/10.1007/s00775-011-0826-3

Homa, J., Rorat, A., Kruk, J., Cocquerelle, C., Plytycz, B., Vandenbulcke, F., 2015. Dermal exposure of *Eisenia andrei* earthworms: Effects of heavy metals on metallothionein and phytochelatin synthase gene expressions in coelomocytes. Environ. Toxicol. Chem. 34, 1397–1404. https://doi.org/10.1002/etc.2944

Hopkin, S.P., 1989. Ecophysiology of metals in terrestrial invertebrates. Ecophysiol. Met. Terr. Invertebr.

Hsiao, I.-L., Hsieh, Y.-K., Wang, C.-F., Chen, I.-C., Huang, Y.-J., 2015. Trojan-Horse Mechanism in the Cellular Uptake of Silver Nanoparticles Verified by Direct Intra- and Extracellular Silver Speciation Analysis. Environ. Sci. Technol. 49, 3813–3821. https://doi.org/10.1021/es504705p

Hullmann, A., 2006. Who is winning the global nanorace? Nat. Nanotechnol. 1, 81–83. https://doi.org/10.1038/nnano.2006.110

INERIS, 2016. Study report N°DRC-16-157037-04739B: Study of the use of nanosilver. URL https://www.ineris.fr/sites/ineris.fr/files/contribution/Documents/r-drc-16-157037-04739b-nano-argent-vf-1483450982.pdf.

Ireland, M.P., 1982. Sites of water, zinc and calcium uptake and distribution of these metals after cadmium administration in *Arion ater* (Gastropoda: Pulmonata). Comp. Biochem. Physiol. A Physiol. 73, 217–221. https://doi.org/10.1016/0300-9629(82)90058-5

ISO 10390:2005, n.d. ISO. URL https://www.iso.org/cms/render/live/fr/sites/isoorg/contents/data/standard/04/08/40879.html.

ISO 15952, 2018. ISO 15952:2018 _ Qualité du sol — Effets des polluants vis-à-vis des escargots juvéniles (Helicidae) — Détermination des effets sur la croissance par contamination du sol.

ISO/TS 80004-1:2015, n.d. ISO/TS 80004-1:2015. ISO. URL https://www.iso.org/cms/render/live/fr/sites/isoorg/contents/data/standard/06/80/68058.html.

Jarwar, A.R., Hao, K., Bitume, E.V., Ullah, H., Cui, D., Nong, X., Wang, G., Tu, X., Zhang, Z., 2019. Comparative Transcriptomic Analysis Reveals Molecular Profiles of Central Nervous System in Maternal Diapause Induction of Locusta migratoria. G3 Genes Genomes Genet. 9, 3287–3296. https://doi.org/10.1534/g3.119.400475

Jaskulak, M., Grobelak, A., Vandenbulcke, F., 2020. Effects of sewage sludge supplementation on heavy metal accumulation and the expression of ABC transporters in *Sinapis alba L.* during assisted phytoremediation of contaminated sites. Ecotoxicol. Environ. Saf. 197, 110606. https://doi.org/10.1016/j.ecoenv.2020.110606

Jesmer, A.H., Velicogna, J.R., Schwertfeger, D.M., Scroggins, R.P., Princz, J.I., 2017. The toxicity of silver to soil organisms exposed to silver nanoparticles and silver nitrate in biosolids-amended field soil: Toxicity of Ag nanoparticles in biosolids-amended soil. Environ. Toxicol. Chem. 36, 2756–2765. https://doi.org/10.1002/etc.3834

Jiravova, J., Tomankova, K.B., Harvanova, M., Malina, L., Malohlava, J., Luhova, L., Panacek, A., Manisova, B., Kolarova, H., 2016. The effect of silver nanoparticles and silver ions on mammalian and plant cells in vitro. Food Chem. Toxicol. 96, 50–61. https://doi.org/10.1016/j.fct.2016.07.015

Jo, P.G., Choi, Y.K., Choi, C.Y., 2008. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 147, 460–469. https://doi.org/10.1016/j.cbpc.2008.02.001

Johnston, H.J., Hutchison, G., Christensen, F.M., Peters, S., Hankin, S., Stone, V., 2010. A review of the in vivo and in vitro toxicity of silver and gold particulates: Particle attributes and biological mechanisms responsible for the observed toxicity. Crit. Rev. Toxicol. 40, 328–346. https://doi.org/10.3109/10408440903453074

Judy, J.D., Kirby, J.K., Creamer, C., McLaughlin, M.J., Fiebiger, C., Wright, C., Cavagnaro, T.R., Bertsch, P.M., 2015. Effects of silver sulfide nanomaterials on mycorrhizal colonization of tomato plants and soil microbial communities in biosolid-amended soil. Environ. Pollut. 206, 256–263. https://doi.org/10.1016/j.envpol.2015.07.002

Kadner, R.J., 1999. Bacteria _ life-form | Cell, Evolution & Classification. Encycl. Br.

Kaegi, R., Sinnet, B., Zuleeg, S., Hagendorfer, H., Mueller, E., Vonbank, R., Boller, M., Burkhardt, M., 2010. Release of silver nanoparticles from outdoor facades. Environ. Pollut. 158, 2900–2905. https://doi.org/10.1016/j.envpol.2010.06.009

Kaegi, R., Voegelin, A., Ort, C., Sinnet, B., Thalmann, B., Krismer, J., Hagendorfer, H., Elumelu, M., Mueller, E., 2013. Fate and transformation of silver nanoparticles in urban wastewater systems. Water Res. 47, 3866–3877. https://doi.org/10.1016/j.watres.2012.11.060

Kaegi, R., Voegelin, A., Sinnet, B., Zuleeg, S., Hagendorfer, H., Burkhardt, M., Siegrist, H., 2011. Behavior of Metallic Silver Nanoparticles in a Pilot Wastewater Treatment Plant. Environ. Sci. Technol. 45, 3902–3908. https://doi.org/10.1021/es1041892

Kampe, S., Kaegi, R., Schlich, K., Wasmuth, C., Hollert, H., Schlechtriem, C., 2018. Silver nanoparticles in sewage sludge: Bioavailability of sulfidized silver to the terrestrial isopod *Porcellio scaber*: Silver nanoparticles in sewage sludge. Environ. Toxicol. Chem. 37, 1606–1613. https://doi.org/10.1002/etc.4102

Kapoor, V., Li, X., Elk, M., Chandran, K., Impellitteri, C.A., Santo Domingo, J.W., 2015. Impact of Heavy Metals on Transcriptional and Physiological Activity of Nitrifying Bacteria. Environ. Sci. Technol. 49, 13454–13462. https://doi.org/10.1021/acs.est.5b02748

Kaveh, R., Li, Y.-S., Ranjbar, S., Tehrani, R., Brueck, C.L., Van Aken, B., 2013. Changes in Arabidopsis thaliana Gene Expression in Response to Silver Nanoparticles and Silver Ions. Environ. Sci. Technol. 47, 10637–10644. https://doi.org/10.1021/es402209w

Kędziora, A., Speruda, M., Krzyżewska, E., Rybka, J., Łukowiak, A., Bugla-Płoskońska, G., 2018. Similarities and Differences between Silver Ions and Silver in Nanoforms as Antibacterial Agents. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19020444

Kent, R.D., Oser, J.G., Vikesland, P.J., 2014. Controlled Evaluation of Silver Nanoparticle Sulfidation in a Full-Scale Wastewater Treatment Plant. Environ. Sci. Technol. 48, 8564–8572. https://doi.org/10.1021/es404989t

Khan, M.R., Rizvi, T.F., 2017. Application of Nanofertilizer and Nanopesticides for Improvements in Crop Production and Protection, in: Ghorbanpour, M., Manika, K., Varma, A. (Eds.), Nanoscience and Plant–Soil Systems, Soil Biology. Springer International Publishing, Cham, pp. 405–427. https://doi.org/10.1007/978-3-319-46835-8_15

Kim, S.W., Nam, S.-H., An, Y.-J., 2012. Interaction of Silver Nanoparticles with Biological Surfaces of *Caenorhabditis elegans*. Ecotoxicol. Environ. Saf. 77, 64–70. https://doi.org/10.1016/j.ecoenv.2011.10.023

Klein, C.L., Stahlmecke, B., Romazanov, J., Kuhlbusch, T.A.J., Van Doren, E., De Temmerman, P.-J., Mast, J., Wick, P., Krug, H., Locoro, G., Hund-Rinke, K., Kördel, W., Friedrichs, S., Maier, G., Werner, J., Linsinger, T., Gawlik, B.M., Comero, S., Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Institute for Environment and Sustainability, Institute for Reference Materials and Measurements, 2011. NM-Series of representative manufactured nanomaterials: NM-300 silver characterisation, stability, homogeneity. Publications Office, Luxembourg.

Kraas, M., Schlich, K., Knopf, B., Wege, F., Kägi, R., Terytze, K., Hund-Rinke, K., 2017. Long-term effects of sulfidized silver nanoparticles in sewage sludge on soil microflora. Environ. Toxicol. Chem. 36, 3305–3313. https://doi.org/10.1002/etc.3904

Krajcarová, L., Novotný, K., Kummerová, M., Dubová, J., Gloser, V., Kaiser, J., 2017. Mapping of the spatial distribution of silver nanoparticles in root tissues of *Vicia faba* by laser-induced breakdown spectroscopy (LIBS). Talanta 173, 28–35. https://doi.org/10.1016/j.talanta.2017.05.055

Kubátová, P., Hejcman, M., Száková, J., Vondráčková, S., Tlustoš, P., 2016. Effects of Sewage Sludge Application on Biomass Production and Concentrations of Cd, Pb and Zn in Shoots of Salix and Populus Clones: Improvement of Phytoremediation Efficiency in Contaminated Soils. BioEnergy Res. 9, 809–819. https://doi.org/10.1007/s12155-016-9727-1

Kumar, N., Palmer, G.R., Shah, V., Walker, V.K., 2014. The Effect of Silver Nanoparticles on Seasonal Change in Arctic Tundra Bacterial and Fungal Assemblages. PLoS ONE 9, e99953. https://doi.org/10.1371/journal.pone.0099953

Kumar, V., Chopra, A.K., Kumar, A., 2017. A Review on Sewage Sludge (Biosolids) a Resource for Sustainable Agriculture. Arch. Agric. Environ. Sci. 2, 340–347. https://doi.org/10.26832/24566632.2017.020417

Lahive, E., Matzke, M., Durenkamp, M., Lawlor, A.J., Thacker, S.A., Pereira, M.G., Spurgeon, D.J., Unrine, J.M., Svendsen, C., Lofts, S., 2017. Sewage sludge treated with metal nanomaterials inhibits earthworm reproduction more strongly than sludge treated with metal metals in bulk/salt forms. Environ. Sci. Nano 4, 78–88. https://doi.org/10.1039/C6EN00280C

Lanier, C., Bernard, F., Dumez, S., Leclercq-Dransart, J., Lemière, S., Vandenbulcke, F., Nesslany, F., Platel, A., Devred, I., Hayet, A., Cuny, D., Deram, A., 2019. Combined toxic effects and DNA damage to two plant species exposed to binary metal mixtures (Cd/Pb). Ecotoxicol. Environ. Saf. 167, 278–287. https://doi.org/10.1016/j.ecoenv.2018.10.010

Lapied, E., Moudilou, E., Exbrayat, J.-M., Oughton, D.H., Joner, E.J., 2010. Silver nanoparticle exposure causes apoptotic response in the earthworm *Lumbricus terrestris* (Oligochaeta). Nanomed. 5, 975–984. https://doi.org/10.2217/nnm.10.58

Layet, C., Santaella, C., Auffan, M., Chevassus-Rosset, C., Montes, M., Levard, C., Ortet, P., Barakat, M., Doelsch, E., 2019. Phytoavailability of silver at predicted environmental concentrations: does the initial ionic or nanoparticulate form matter? Environ. Sci. Nano 6, 127–135. https://doi.org/10.1039/C8EN00644J

Lead, J.R., Batley, G.E., Alvarez, P.J.J., Croteau, M.-N., Handy, R.D., McLaughlin, M.J., Judy, J.D., Schirmer, K., 2018. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects-An updated review: Nanomaterials in the environment. Environ. Toxicol. Chem. 37, 2029–2063. https://doi.org/10.1002/etc.4147

Levard, C., Hotze, E.M., Colman, B.P., Dale, A.L., Truong, L., Yang, X.Y., Bone, A.J., Brown, G.E., Tanguay, R.L., Di Giulio, R.T., Bernhardt, E.S., Meyer, J.N., Wiesner, M.R., Lowry, G.V., 2013. Sulfidation of Silver Nanoparticles: Natural Antidote to Their Toxicity. Environ. Sci. Technol. 47, 13440–13448. https://doi.org/10.1021/es403527n

Levard, C., Hotze, E.M., Lowry, G.V., Brown, G.E., 2012. Environmental Transformations of Silver Nanoparticles: Impact on Stability and Toxicity. Environ. Sci. Technol. 46, 6900–6914. https://doi.org/10.1021/es2037405

Levard, C., Reinsch, B.C., Michel, F.M., Oumahi, C., Lowry, G.V., Brown, G.E., 2011. Sulfidation Processes of PVP-Coated Silver Nanoparticles in Aqueous Solution: Impact on Dissolution Rate. Environ. Sci. Technol. 45, 5260–5266. https://doi.org/10.1021/es2007758

Lim, D., Roh, J., Eom, H., Choi, J.-Y., Hyun, J., Choi, J., 2012. Oxidative stress-related PMK-1 P38 MAPK activation as a mechanism for toxicity of silver nanoparticles to reproduction in the nematode *Caenorhabditis elegans*. Environ. Toxicol. Chem. 31, 585–592. https://doi.org/10.1002/etc.1706

Limpiteeprakan, P., Babel, S., Lohwacharin, J., Takizawa, S., 2016. Release of silver nanoparticles from fabrics during the course of sequential washing. Environ. Sci. Pollut. Res. 23, 22810–22818. https://doi.org/10.1007/s11356-016-7486-3

Liu, G., Zhang, M., Jin, Y., Fan, X., Xu, J., Zhu, Y., Fu, Z., Pan, X., Qian, H., 2017. The Effects of Low Concentrations of Silver Nanoparticles on Wheat Growth, Seed Quality, and Soil Microbial Communities. Water. Air. Soil Pollut. 228, 348. https://doi.org/10.1007/s11270-017-3523-1

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lombi, E., Donner, E., Taheri, S., Tavakkoli, E., Jämting, Å.K., McClure, S., Naidu, R., Miller, B.W., Scheckel, K.G., Vasilev, K., 2013. Transformation of four silver/silver chloride nanoparticles during anaerobic treatment of wastewater and post-processing of sewage sludge. Environ. Pollut. 176, 193–197. https://doi.org/10.1016/j.envpol.2013.01.029

Lubbers, I.M., van Groenigen, K.J., Fonte, S.J., Six, J., Brussaard, L., van Groenigen, J.W., 2013. Greenhouse-gas emissions from soils increased by earthworms. Nat. Clim. Change 3, 187–194. https://doi.org/10.1038/nclimate1692

Luo, X., Xu, S., Yang, Y., Li, L., Chen, S., Xu, A., Wu, L., 2016. Insights into the Ecotoxicity of Silver Nanoparticles Transferred from *Escherichia coli* to *Caenorhabditis elegans*. Sci. Rep. 6, 36465. https://doi.org/10.1038/srep36465

Ma, J.F., Yamaji, N., 2006. Silicon uptake and accumulation in higher plants. Trends Plant Sci. 11, 392–397. https://doi.org/10.1016/j.tplants.2006.06.007

Ma, R., Levard, C., Judy, J.D., Unrine, J.M., Durenkamp, M., Martin, B., Jefferson, B., Lowry, G.V., 2014. Fate of Zinc Oxide and Silver Nanoparticles in a Pilot Wastewater Treatment Plant and in Processed Biosolids. Environ. Sci. Technol. 48, 104–112. https://doi.org/10.1021/es403646x

Ma, R., Levard, C., Marinakos, S.M., Cheng, Y., Liu, J., Michel, F.M., Brown, G.E., Lowry, G.V., 2012. Size-Controlled Dissolution of Organic-Coated Silver Nanoparticles. Environ. Sci. Technol. 46, 752–759. https://doi.org/10.1021/es201686j

Mahé, F., Rognes, T., Quince, C., Vargas, C. de, Dunthorn, M., 2014. Swarm: robust and fast clustering method for amplicon-based studies. PeerJ 2, e593. https://doi.org/10.7717/peerj.593

Maillard, J.-Y., Hartemann, P., 2013. Silver as an antimicrobial: facts and gaps in knowledge. Crit. Rev. Microbiol. 39, 373–383. https://doi.org/10.3109/1040841X.2012.713323

Makama, S., Piella, J., Undas, A., Dimmers, W.J., Peters, R., Puntes, V.F., van den Brink, N.W., 2016. Properties of silver nanoparticles influencing their uptake in and toxicity to the earthworm *Lumbricus rubellus* following exposure in soil. Environ. Pollut. 218, 870–878. https://doi.org/10.1016/j.envpol.2016.08.016

Mariet, A.-L., Pauget, B., de Vaufleury, A., Bégeot, C., Walter-Simonnet, A.-V., Gimbert, F., 2017. Using bioindicators to assess the environmental risk of past mining activities in the Vosges Mountains (France). Ecol. Indic. 75, 17–26. https://doi.org/10.1016/j.ecolind.2016.11.042

Mariyadas, J., Amorim, M.J.B., Jensen, J., Scott-Fordsmand, J.J., 2018. Earthworm avoidance of silver nanomaterials over time. Environ. Pollut. 239, 751–756. https://doi.org/10.1016/j.envpol.2018.04.059

Massarsky, A., Trudeau, V.L., Moon, T.W., 2014. Predicting the environmental impact of nanosilver. Environ. Toxicol. Pharmacol. 38, 861–873. https://doi.org/10.1016/j.etap.2014.10.006

Maurer, L.L., Yang, X., Schindler, A.J., Taggart, R.K., Jiang, C., Hsu-Kim, H., Sherwood, D.R., Meyer, J.N., 2016. Intracellular trafficking pathways in silver nanoparticle uptake and toxicity in *Caenorhabditis elegans*. Nanotoxicology 10, 831–835. https://doi.org/10.3109/17435390.2015.1110759

McGee, C.F., Storey, S., Clipson, N., Doyle, E., 2017. Soil microbial community responses to contamination with silver, aluminium oxide and silicon dioxide nanoparticles. Ecotoxicology 26, 449–458. https://doi.org/10.1007/s10646-017-1776-5

McGillicuddy, E., Murray, I., Kavanagh, S., Morrison, L., Fogarty, A., Cormican, M., Dockery, P., Prendergast, M., Rowan, N., Morris, D., 2017. Silver nanoparticles in the environment: Sources, detection and ecotoxicology. Sci. Total Environ. 575, 231–246. https://doi.org/10.1016/j.scitotenv.2016.10.041

McKee, M.S., Engelke, M., Zhang, X., Lesnikov, E., Köser, J., Eickhorst, T., Filser, J., 2017. Collembola Reproduction Decreases with Aging of Silver Nanoparticles in a Sewage

Sludge-Treated Soil. Front. Environ. Sci. 5, 19. https://doi.org/10.3389/fenvs.2017.00019

McKee, M.S., Köser, J., Focke, O., Filser, J., 2019. A new test system for unraveling the effects of soil components on the uptake and toxicity of silver nanoparticles (NM-300K) in simulated pore water. Sci. Total Environ. 673, 613–621. https://doi.org/10.1016/j.scitotenv.2019.03.493

Mendes, L.A., Maria, V.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Ag Nanoparticles (Ag NM300K) in the Terrestrial Environment: Effects at Population and Cellular Level in *Folsomia candida* (Collembola). Int. J. Environ. Res. Public. Health 12, 12530–12542. https://doi.org/10.3390/ijerph121012530

Michels, C., Perazzoli, S., M. Soares, H., 2017. Inhibition of an enriched culture of ammonia oxidizing bacteria by two different nanoparticles: Silver and magnetite. Sci. Total Environ. 586, 995–1002. https://doi.org/10.1016/j.scitotenv.2017.02.080

Michels, C., Yang, Y., Moreira Soares, H., Alvarez, P.J.J., 2015. Silver nanoparticles temporarily retard NO2 − production without significantly affecting $N_2O$ release by *Nitrosomonas europaea*: AgNPs hinder *Nitrosomonas europaea* activity. Environ. Toxicol. Chem. 34, 2231–2235. https://doi.org/10.1002/etc.3071

Mirzajani, F., Askari, H., Hamzelou, S., Farzaneh, M., Ghassempour, A., 2013. Effect of silver nanoparticles on *Oryza sativa L.* and its rhizosphere bacteria. Ecotoxicol. Environ. Saf. 88, 48–54. https://doi.org/10.1016/j.ecoenv.2012.10.018

Morgan, A.J., Stürzenbaum, S.R., Winters, C., Grime, G.W., Aziz, N.A.A., Kille, P., 2004. Differential metallothionein expression in earthworm (*Lumbricus rubellus*) tissues. Ecotoxicol. Environ. Saf. 57, 11–19. https://doi.org/10.1016/j.ecoenv.2003.08.022

Morgan, J.E., Morgan, A.J., 1993. Seasonal changes in the tissue-metal (Cd, Zn and Pb) concentrations in two ecophysiologically dissimilar earthworm species: pollution-monitoring implications. Environ. Pollut. 82, 1–7. https://doi.org/10.1016/0269-7491(93)90155-H

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramírez, J.T., Yacaman, M.J., 2005. The bactericidal effect of silver nanoparticles. Nanotechnology 16, 2346–2353. https://doi.org/10.1088/0957-4484/16/10/059

Nair, P.M.G., Chung, I.M., 2015. Physiological and molecular level studies on the toxicity of silver nanoparticles in germinating seedlings of mung bean (*Vigna radiata L.*). Acta Physiol. Plant. 37, 1719. https://doi.org/10.1007/s11738-014-1719-1

Nair, P.M.G., Chung, I.M., 2014. Assessment of silver nanoparticle-induced physiological and molecular changes in *Arabidopsis thaliana*. Environ. Sci. Pollut. Res. 21, 8858–8869. https://doi.org/10.1007/s11356-014-2822-y

Nordberg, G.F., 1989. Modulation of metal toxicity by metallothionein. Biol. Trace Elem. Res. 21, 131. https://doi.org/10.1007/BF02917245

Novo, M., Lahive, E., Díez-Ortiz, M., Matzke, M., Morgan, A.J., Spurgeon, D.J., Svendsen, C., Kille, P., 2015. Different routes, same pathways: Molecular mechanisms under silver ion and nanoparticle exposures in the soil sentinel *Eisenia fetida*. Environ. Pollut. 205, 385–393. https://doi.org/10.1016/j.envpol.2015.07.010

Nowack, B., Krug, H.F., Height, M., 2011. 120 Years of Nanosilver History: Implications for Policy Makers. Environ. Sci. Technol. 45, 1177–1183. https://doi.org/10.1021/es103316q

Nowack, B., Mitrano, D.M., 2018. Procedures for the production and use of synthetically aged and product released nanomaterials for further environmental and ecotoxicity testing. NanoImpact 10, 70–80. https://doi.org/10.1016/j.impact.2017.12.001

OCDE, 1984. Ver de terre, essai de toxicité aigüe, Ligne directrice N°207, Ligne directrice de l'OCDE pour les essais de produits chimiques, OCDE, Paris.

O'Hara, G.W., Daniel, R.M., 1985. Rhizobial denitrification: A review. Soil Biol. Biochem. 17, 1–9. https://doi.org/10.1016/0038-0717(85)90082-3

Ojha, R.B., Devkota, D., 2014. Earthworms: "Soil and Ecosystem Engineers" – a Review. World J. Agric. Res. 2, 257–260. https://doi.org/10.12691/wjar-2-6-1

Olchowik, J., Bzdyk, R., Studnicki, M., Bederska-Błaszczyk, M., Urban, A., Aleksandrowicz-Trzcińska, M., 2017. The Effect of Silver and Copper Nanoparticles on the Condition of English Oak (*Quercus robur L.*) Seedlings in a Container Nursery Experiment. Forests 8, 310. https://doi.org/10.3390/f8090310

Patra, A.K., Abbadie, L., Clays-Josserand, A., Degrange, V., Grayston, S.J., Loiseau, P., Louault, F., Mahmood, S., Nazaret, S., Philippot, L., Poly, F., Prosser, J.I., Richaume, A., Roux, X.L., 2005. Effects of Grazing on Microbial Functional Groups Involved in Soil N Dynamics. Ecol. Monogr. 75, 65–80. https://doi.org/10.1890/03-0837

Pauget, B., Faure, O., Conord, C., Crini, N., de Vaufleury, A., 2015. In situ assessment of phyto and zooavailability of trace elements: A complementary approach to chemical extraction procedures. Sci. Total Environ. 521–522, 400–410. https://doi.org/10.1016/j.scitotenv.2015.03.075

Pauget, B., Gimbert, F., Scheifler, R., Coeurdassier, M., De Vaufleury, A., 2012. Soil parameters are key factors to predict metal bioavailability to snails based on chemical extractant data. Sci. Total Environ. 431, 413–425. https://doi.org/10.1016/j.scitotenv.2012.05.048

Peyrot, C., Wilkinson, K.J., Desrosiers, M., Sauvé, S., 2014. Effects of silver nanoparticles on soil enzyme activities with and without added organic matter: Effects of silver nanoparticles on soil enzyme activities. Environ. Toxicol. Chem. 33, 115–125. https://doi.org/10.1002/etc.2398

Pittol, M., Tomacheski, D., Simões, D.N., Ribeiro, V.F., Santana, R.M.C., 2017. Macroscopic effects of silver nanoparticles and titanium dioxide on edible plant growth. Environ. Nanotechnol. Monit. Manag. 8, 127–133. https://doi.org/10.1016/j.enmm.2017.07.003

Poma, G., Cuykx, M., Amato, E., Calaprice, C., Focant, J.F., Covaci, A., 2017. Evaluation of hazardous chemicals in edible insects and insect-based food intended for human consumption. Food Chem. Toxicol. 100, 70–79. https://doi.org/10.1016/j.fct.2016.12.006

Pradas del Real, A.E., Castillo-Michel, H., Kaegi, R., Sinnet, B., Magnin, V., Findling, N., Villanova, J., Carrière, M., Santaella, C., Fernández-Martínez, A., Levard, C., Sarret, G., 2016. Fate of Ag-NPs in Sewage Sludge after Application on Agricultural Soils. Environ. Sci. Technol. 50, 1759–1768. https://doi.org/10.1021/acs.est.5b04550

Pradas del Real, A.E., Vidal, V., Carrière, M., Castillo-Michel, H., Levard, C., Chaurand, P., Sarret, G., 2017. Silver Nanoparticles and Wheat Roots: A Complex Interplay. Environ. Sci. Technol. 51, 5774–5782. https://doi.org/10.1021/acs.est.7b00422

Proux, O., Biquard, X., Lahera, E., Menthonnex, J.-J., Prat, A., Ulrich, O., Soldo, Y., Trévisson, P., Kapoujyan, G., Perroux, G., Taunier, P., Grand, D., Jeantet, P., Deléglise, M., Roux,

J.-P., Hazemann, J.-L., 2005. FAME : A new beamline for X-ray absorption investigations of very-diluted systems of environmental, material and biological interests. Phys. Scr. 115, 970–973. https://doi.org/10.1238/Physica.Topical.115a00970

Qian, H., Peng, X., Han, X., Ren, J., Sun, L., Fu, Z., 2013. Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. J. Environ. Sci. 25, 1947–1956. https://doi.org/10.1016/S1001-0742(12)60301-5

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219

R Core Team, 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Radwan, M.A., El-Gendy, K.S., Gad, A.F., Khamis, A.E., Eshra, E.H., 2019. Responses of oxidative stress, genotoxicity and immunotoxicity as biomarkers in *Theba pisana* snails dietary exposed to silver nanoparticles. Chem. Ecol. 35, 613–630. https://doi.org/10.1080/02757540.2019.1631816

Rahmatpour, S., Shirvani, M., Mosaddeghi, M.R., Nourbakhsh, F., Bazarganipour, M., 2017. Dose–response effects of silver nanoparticles and silver nitrate on microbial and enzyme activities in calcareous soils. Geoderma 285, 313–322. https://doi.org/10.1016/j.geoderma.2016.10.006

Rai, M., Yadav, A., Gade, A., 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27, 76–83. https://doi.org/10.1016/j.biotechadv.2008.09.002

Rasool, K., Lee, D.S., 2016. Inhibitory Effects of Silver Nanoparticles on Removal of Organic Pollutants and Sulfate in an Anaerobic Biological Wastewater Treatment Process. J. Nanosci. Nanotechnol. 16, 4456–4463. https://doi.org/10.1166/jnn.2016.10984

Ravel, B., Newville, M., 2005. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. J. Synchrotron Radiat. 12, 537–541. https://doi.org/10.1107/S0909049505012719

Rawlings, D.E., Kusano, T., 1994. Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. 58, 39–55.

Regulation n° 1881/2006 of European Commission, 2006. Réglement (CE) N°1881/2006 de la commission du 19 décembre 2006 portant fixation de teneurs maximales pour certains contaminants dans les denrées alimentaires.

Reidy, B., Haase, A., Luch, A., Dawson, K., Lynch, I., 2013. Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. Materials 6, 2295–2350. https://doi.org/10.3390/ma6062295

Reinsch, B.C., Levard, C., Li, Z., Ma, R., Wise, A., Gregory, K.B., Brown, G.E., Lowry, G.V., 2012. Sulfidation of silver nanoparticles decreases *Escherichia coli* growth inhibition. Environ. Sci. Technol. 46, 6992–7000. https://doi.org/10.1021/es203732x

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584. https://doi.org/10.7717/peerj.2584

Roh, J., Sim, S.J., Yi, J., Park, K., Chung, K.H., Ryu, D., Choi, J., 2009. Ecotoxicity of Silver Nanoparticles on the Soil Nematode *Caenorhabditis elegans* Using Functional Ecotoxicogenomics. Environ. Sci. Technol. 43, 3933–3940. https://doi.org/10.1021/es803477u

Rorat, A., 2015. Assessment of the vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses ofthree earthworm species: Eisenia fetida, Eisenia andrei and Dendrobaena veneta.

Rorat, A., Courtois, P., Vandenbulcke, F., Lemiere, S., 2019. 8 - Sanitary and environmental aspects of sewage sludge management, in: Prasad, M.N.V., de Campos Favas, P.J., Vithanage, M., Mohan, S.V. (Eds.), Industrial and Municipal Sludge. Butterworth-Heinemann, pp. 155–180. https://doi.org/10.1016/B978-0-12-815907-1.00008-8

Rorat, A., Vandenbulcke, F., Gałuszka, A., Klimek, B., Plytycz, B., 2017. Protective role of metallothionein during regeneration in *Eisenia andrei* exposed to cadmium. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 203, 39–50. https://doi.org/10.1016/j.cbpc.2017.10.002

Roubalová, R., Płytycz, B., Procházková, P., Navarro Pacheco, N.I., Bilej, M., 2018. Annelida: Environmental Interactions and Ecotoxicity in Relation to the Earthworm Immune System, in: Cooper, E.L. (Ed.), Advances in Comparative Immunology. Springer International Publishing, Cham, pp. 933–951. https://doi.org/10.1007/978-3-319-76768-0_27

Rui, M., Ma, C., Tang, X., Yang, J., Jiang, F., Pan, Y., Xiang, Z., Hao, Y., Rui, Y., Cao, W., Xing, B., 2017. Phytotoxicity of Silver Nanoparticles to Peanut (*Arachis hypogaea L.*): Physiological Responses and Food Safety. ACS Sustain. Chem. Eng. 5, 6557–6567. https://doi.org/10.1021/acssuschemeng.7b00736

Sadak, M.S., 2019. Impact of silver nanoparticles on plant growth, some biochemical aspects, and yield of fenugreek plant (*Trigonella foenum-graecum*). Bull. Natl. Res. Cent. 43, 38. https://doi.org/10.1186/s42269-019-0077-y

Samarajeewa, A.D., Velicogna, J.R., Princz, J.I., Subasinghe, R.M., Scroggins, R.P., Beaudette, L.A., 2017. Effect of silver nano-particles on soil microbial growth, activity and community diversity in a sandy loam soil. Environ. Pollut. 220, 504–513. https://doi.org/10.1016/j.envpol.2016.09.094

Samarajeewa, A.D., Velicogna, J.R., Schwertfeger, D.M., Jesmer, A.H., Princz, J.I., Subasinghe, R.M., Scroggins, R.P., Beaudette, L.A., 2019. Effect of silver nanoparticle contaminated biosolids on the soil microbial community. NanoImpact 14, 100157. https://doi.org/10.1016/j.impact.2019.100157

Schlich, K., Beule, L., Hund-Rinke, K., 2016. Single versus repeated applications of CuO and Ag nanomaterials and their effect on soil microflora. Environ. Pollut. 215, 322–330. https://doi.org/10.1016/j.envpol.2016.05.028

Schlich, K., Hoppe, M., Kraas, M., Fries, E., Hund-Rinke, K., 2017. Ecotoxicity and fate of a silver nanomaterial in an outdoor lysimeter study. Ecotoxicology 26, 738–751. https://doi.org/10.1007/s10646-017-1805-4

Schlich, K., Hoppe, M., Kraas, M., Schubert, J., Chanana, M., Hund-Rinke, K., 2018. Long-term effects of three different silver sulfide nanomaterials, silver nitrate and bulk silver sulfide on soil microorganisms and plants. Environ. Pollut. 242, 1850–1859. https://doi.org/10.1016/j.envpol.2018.07.082

Schlich, K., Hund-Rinke, K., 2015. Influence of soil properties on the effect of silver nanomaterials on microbial activity in five soils. Environ. Pollut. 196, 321–330. https://doi.org/10.1016/j.envpol.2014.10.021

Schlich, K., Klawonn, T., Terytze, K., Hund-Rinke, K., 2013. Effects of silver nanoparticles and silver nitrate in the earthworm reproduction test. Environ. Toxicol. Chem. 32, 181–188. https://doi.org/10.1002/etc.2030

Schultz, C.L., Wamucho, A., Tsyusko, O.V., Unrine, J.M., Crossley, A., Svendsen, C., Spurgeon, D.J., 2016. Multigenerational exposure to silver ions and silver nanoparticles reveals heightened sensitivity and epigenetic memory in *Caenorhabditis elegans*. Proc. R. Soc. B Biol. Sci. 283, 20152911. https://doi.org/10.1098/rspb.2015.2911

Sekine, R., Brunetti, G., Donner, E., Khaksar, M., Vasilev, K., Jämting, Å.K., Scheckel, K.G., Kappen, P., Zhang, H., Lombi, E., 2015. Speciation and Lability of Ag-, AgCl-, and $Ag_2S$-Nanoparticles in Soil Determined by X-ray Absorption Spectroscopy and Diffusive Gradients in Thin Films. Environ. Sci. Technol. 49, 897–905. https://doi.org/10.1021/es504229h

Shi, E., Xu, Z., Zhang, X., Yang, X., Liu, Q., Zhang, H., Wimmer, A., Li, L., 2018. Re-evaluation of stability and toxicity of silver sulfide nanoparticle in environmental water: Oxidative dissolution by manganese oxide. Environ. Pollut. 243, 1242–1251. https://doi.org/10.1016/j.envpol.2018.09.103

Shin, Y.-J., Kwak, J.I., An, Y.-J., 2012. Evidence for the inhibitory effects of silver nanoparticles on the activities of soil exoenzymes. Chemosphere 88, 524–529. https://doi.org/10.1016/j.chemosphere.2012.03.010

Shoults-Wilson, W.A., Reinsch, B.C., Tsyusko, O.V., Bertsch, P.M., Lowry, G.V., Unrine, J.M., 2010. Effect of silver nanoparticle surface coating on bioaccumulation and reproductive toxicity in earthworms (*Eisenia fetida*). Nanotoxicology 5, 432–444. https://doi.org/10.3109/17435390.2010.537382

Shoults-Wilson, W.A., Zhurbich, O.I., McNear, D.H., Tsyusko, O.V., Bertsch, P.M., Unrine, J.M., 2011. Evidence for avoidance of Ag nanoparticles by earthworms (*Eisenia fetida*). Ecotoxicology 20, 385–396. https://doi.org/10.1007/s10646-010-0590-0

Sillen, W.M.A., Thijs, S., Abbamondi, G.R., Janssen, J., Weyens, N., White, J.C., Vangronsveld, J., 2015. Effects of silver nanoparticles on soil microorganisms and maize biomass are linked in the rhizosphere. Soil Biol. Biochem. 91, 14–22. https://doi.org/10.1016/j.soilbio.2015.08.019

Smith, M.S., Tiedje, J.M., 1979. Phases of denitrification following oxygen depletion in soil 11, 261–267.

Smorczewski, W.T., Schmidt, E.L., 1991. Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment. Can. J. Microbiol. 37, 828–833. https://doi.org/10.1139/m91-143

Soliman, M., El-Shazly, M., 2017. Bioaccumulation of Heavy Metals by Grasshoppers and a Mantid Along a Pollution Gradient. Ecol. Balk. 9, 7–21.

Song, U., Jun, H., Waldman, B., Roh, J., Kim, Y., Yi, J., Lee, E.J., 2013. Functional analyses of nanoparticle toxicity: A comparative study of the effects of $TiO_2$ and Ag on tomatoes (*Lycopersicon esculentum*). Ecotoxicol. Environ. Saf. 93, 60–67. https://doi.org/10.1016/j.ecoenv.2013.03.033

Spurgeon, D.J., Hopkin, S.P., 1996. The effects of metal contamination on earthworm populations around a smelting works: quantifying species effects. Appl. Soil Ecol. 4, 147–160. https://doi.org/10.1016/0929-1393(96)00109-6

Stampoulis, D., Sinha, S.K., White, J.C., 2009. Assay-Dependent Phytotoxicity of Nanoparticles to Plants. Environ. Sci. Technol. 43, 9473–9479. https://doi.org/10.1021/es901695c

Starnes, D.L., Lichtenberg, S.S., Unrine, J.M., Starnes, C.P., Oostveen, E.K., Lowry, G.V., Bertsch, P.M., Tsyusko, O.V., 2016. Distinct transcriptomic responses of *Caenorhabditis elegans* to pristine and sulfidized silver nanoparticles. Environ. Pollut. 213, 314–321. https://doi.org/10.1016/j.envpol.2016.01.020

Starnes, D.L., Unrine, J.M., Starnes, C.P., Collin, B.E., Oostveen, E.K., Ma, R., Lowry, G.V., Bertsch, P.M., Tsyusko, O.V., 2015. Impact of sulfidation on the bioavailability and toxicity of silver nanoparticles to *Caenorhabditis elegans*. Environ. Pollut. 196, 239–246. https://doi.org/10.1016/j.envpol.2014.10.009

Stegemeier, J.P., Schwab, F., Colman, B.P., Webb, S.M., Newville, M., Lanzirotti, A., Winkler, C., Wiesner, M.R., Lowry, G.V., 2015. Speciation Matters: Bioavailability of Silver and Silver Sulfide Nanoparticles to Alfalfa (*Medicago sativa*). Environ. Sci. Technol. 49, 8451–8460. https://doi.org/10.1021/acs.est.5b01147

Sugawara, N., Sugawara, C., 1984. Comparative study of effect of acute administration of cadmium and silver on ceruloplasmin and metallothionein: Involvement of disposition of copper, iron, and zinc. Environ. Res. 35, 507–515. https://doi.org/10.1016/0013-9351(84)90157-9

Suleiman, H., Rorat, A., Grobelak, A., Grosser, A., Milczarek, M., Płytycz, B., Kacprzak, M., Vandenbulcke, F., 2017. Determination of the performance of vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses in three earthworm species: *Eisenia fetida*, *Eisenia andrei* and *Dendrobaena veneta*. Bioresour. Technol. 241, 103–112. https://doi.org/10.1016/j.biortech.2017.05.104

Sun, J., Wang, L., Li, S., Yin, L., Huang, J., Chen, C., 2017. Toxicity of silver nanoparticles to Arabidopsis : Inhibition of root gravitropism by interfering with auxin pathway: AgNPs inhibit Arabidopsis root gravitropism. Environ. Toxicol. Chem. 36, 2773–2780. https://doi.org/10.1002/etc.3833

The Royal Society, The Royal Academy of Engineering, 2004. Nanoscience and nanotechnologies: opportunities and uncertainties. Royal Society : Royal Academy of Engineering, London.

Thuesombat, P., Hannongbua, S., Akasit, S., Chadchawan, S., 2014. Effect of silver nanoparticles on rice (Oryza sativa L. cv. KDML 105) seed germination and seedling growth. Ecotoxicol. Environ. Saf. 104, 302–309. https://doi.org/10.1016/j.ecoenv.2014.03.022

Tiede, K., Boxall, A.B.A., Wang, X., Gore, D., Tiede, D., Baxter, M., David, H., Tear, S.P., Lewis, J., 2010. Application of hydrodynamic chromatography-ICP-MS to investigate the fate of silver nanoparticles in activated sludge. J. Anal. At. Spectrom. 25, 1149. https://doi.org/10.1039/b926029c

Topuz, E., van Gestel, C.A.M., 2017. The effect of soil properties on the toxicity and bioaccumulation of Ag nanoparticles and Ag ions in *Enchytraeus crypticus*. Ecotoxicol. Environ. Saf. 144, 330–337. https://doi.org/10.1016/j.ecoenv.2017.06.037

Tourinho, P.S., van Gestel, C.A.M., Morgan, A.J., Kille, P., Svendsen, C., Jurkschat, K., Mosselmans, J.F.W., Soares, A.M.V.M., Loureiro, S., 2016. Toxicokinetics of Ag in the terrestrial isopod *Porcellionides pruinosus* exposed to Ag NPs and $AgNO_3$ via soil and food. Ecotoxicology 25, 267–278. https://doi.org/10.1007/s10646-015-1585-7

Tsyusko, O.V., Hardas, S.S., Shoults-Wilson, W.A., Starnes, C.P., Joice, G., Butterfield, D.A., Unrine, J.M., 2012. Short-term molecular-level effects of silver nanoparticle exposure on the earthworm, *Eisenia fetida*. Environ. Pollut. 171, 249–255. https://doi.org/10.1016/j.envpol.2012.08.003

Unrine, J., Bertsch, P., Hunyadi, S., 2008. Bioavailability, Trophic Transfer, and Toxicity of Manufactured Metal and Metal Oxide Nanoparticles in Terrestrial Environments, in: Nanoscience and Nanotechnology. John Wiley & Sons, Ltd, pp. 345–366. https://doi.org/10.1002/9780470396612.ch14

U.S. EPA, U.S.E.P.A., 2009. Targeted National Sewage Sludge Survey Statistical Analysis Report.

Usman, K., Khan, S., Ghulam, S., Khan, M.U., Khan, N., Khan, M.A., Khalil, S.K., 2012. Sewage Sludge: An Important Biological Resource for Sustainable Agriculture and Its Environmental Implications. Am. J. Plant Sci. 03, 1708–1721. https://doi.org/10.4236/ajps.2012.312209

Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780. https://doi.org/10.3762/bjnano.6.181

Velicogna, J.R., Ritchie, E.E., Scroggins, R.P., Princz, J.I., 2016. A comparison of the effects of silver nanoparticles and silver nitrate on a suite of soil dwelling organisms in two field soils. Nanotoxicology 10, 1144–1151. https://doi.org/10.1080/17435390.2016.1181807

Velicogna, J.R., Schwertfeger, D.M., Jesmer, A.H., Scroggins, R.P., Princz, J.I., 2017. The bioaccumulation of silver in *Eisenia andrei* exposed to silver nanoparticles and silver nitrate in soil. NanoImpact 6, 11–18. https://doi.org/10.1016/j.impact.2017.03.001

Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W.J.G.M., 2004. Internal metal sequestration and its ecotoxicological relevance: a review. Environ. Sci. Technol. 38, 4705–4712. https://doi.org/10.1021/es040354g

Vijver, M.G., Vink, J.P.M., Miermans, C.J.H., van Gestel, C.A.M., 2003. Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworms. Soil Biol. Biochem. 35, 125–132. https://doi.org/10.1016/S0038-0717(02)00245-6

Vishwakarma, K., Shweta, Upadhyay, N., Singh, J., Liu, S., Singh, V.P., Prasad, S.M., Chauhan, D.K., Tripathi, D.K., Sharma, S., 2017. Differential Phytotoxic Impact of Plant Mediated Silver Nanoparticles (AgNPs) and Silver Nitrate ($AgNO_3$) on *Brassica sp.* Front. Plant Sci. 8, 1501. https://doi.org/10.3389/fpls.2017.01501

Vitez, T., Novak, D., Lochman, J., Vitezova, M., 2020. Methanogens Diversity during Anaerobic Sewage Sludge Stabilization and the Effect of Temperature 822.

Vittori Antisari, L., Carbone, S., Gatti, A., Ferrando, S., Nacucchi, M., Pascalis, F.D., Gambardella, C., Badalucco, L., Laudicina, V.A., 2016. Effect of cobalt and silver nanoparticles and ions on *Lumbricus rubellus* health and on microbial community of

earthworm faeces and soil. Appl. Soil Ecol. 108, 62–71. https://doi.org/10.1016/j.apsoil.2016.07.019

Walter, A., Probst, M., Franke-Whittle, I.H., Ebner, C., Podmirseg, S.M., Etemadi-Shalamzari, M., Hupfauf, S., Insam, H., 2019. Microbiota in anaerobic digestion of sewage sludge with and without co-substrates. Water Environ. J. 33, 214–222. https://doi.org/10.1111/wej.12392

Wang, J., Shu, K., Zhang, L., Si, Y., 2017. Effects of Silver Nanoparticles on Soil Microbial Communities and Bacterial Nitrification in Suburban Vegetable Soils. Pedosphere 27, 482–490. https://doi.org/10.1016/S1002-0160(17)60344-8

Wang, P., Lombi, E., Sun, S., Scheckel, K.G., Malysheva, A., McKenna, B.A., Menzies, N.W., Zhao, F.-J., Kopittke, P.M., 2017. Characterizing the uptake, accumulation and toxicity of silver sulfide nanoparticles in plants. Environ. Sci. Nano 4, 448–460. https://doi.org/10.1039/C6EN00489J

Wang, P., Menzies, N.W., Lombi, E., Sekine, R., Blamey, F.P.C., Hernandez-Soriano, M.C., Cheng, M., Kappen, P., Peijnenburg, W.J.G.M., Tang, C., Kopittke, P.M., 2015. Silver sulfide nanoparticles ($Ag_2S$-NPs) are taken up by plants and are phytotoxic. Nanotoxicology 9, 1041–1049. https://doi.org/10.3109/17435390.2014.999139

Wang, X.-T., Xu, X., Chen, C., Xing, D., Zhang, R.-C., Zhou, X., Yuan, Y., Wang, A.-J., Lee, D.-J., 2019. The microbial zonation of SRB and soNRB enhanced the performance of SR-DSR process under the micro-aerobic condition. Environ. Int. 132, 105096. https://doi.org/10.1016/j.envint.2019.105096

Wertz, S., Degrange, V., Prosser, J.I., Poly, F., Commeaux, C., Guillaumaud, N., Roux, X.L., 2007. Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. Environ. Microbiol. 9, 2211–2219. https://doi.org/10.1111/j.1462-2920.2007.01335.x

Whitley, A.R., Levard, C., Oostveen, E., Bertsch, P.M., Matocha, C.J., Kammer, F. von der, Unrine, J.M., 2013. Behavior of Ag nanoparticles in soil: Effects of particle surface coating, aging and sewage sludge amendment. Environ. Pollut. 182, 141–149. https://doi.org/10.1016/j.envpol.2013.06.027

Wijnhoven, S.W.P., Peijnenburg, W.J.G.M., Herberts, C.A., Hagens, W.I., Oomen, A.G., Heugens, E.H.W., Roszek, B., Bisschops, J., Gosens, I., Van De Meent, D., Dekkers, S., De Jong, W.H., van Zijverden, M., Sips, A.J.A.M., Geertsma, R.E., 2009. Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology 3, 109–138. https://doi.org/10.1080/17435390902725914

Wu, J., Bai, Y., Lu, B., Zhao, W., Forstner, C., Menzies, N.W., Bertsch, P.M., Wang, P., Kopittke, P.M., 2020. Silver Sulfide Nanoparticles Reduce Nitrous Oxide Emissions by Inhibiting Denitrification in the Earthworm Gut. Environ. Sci. Technol. 54, 11146–11154. https://doi.org/10.1021/acs.est.0c01241

Wu, J.-W., Shi, Y., Zhu, Y.-X., Wang, Y.-C., Gong, H.-J., 2013. Mechanisms of Enhanced Heavy Metal Tolerance in Plants by Silicon: A Review. Pedosphere 23, 815–825. https://doi.org/10.1016/S1002-0160(13)60073-9

Wu, L., Yang, L., Wang, Z., Cheng, M., Li, Z., Liu, W., Ma, T., Christie, P., Luo, Y., 2018. Uptake of silver by brown rice and wheat in soils repeatedly amended with biosolids. Sci. Total Environ. 612, 94–102. https://doi.org/10.1016/j.scitotenv.2017.08.183

Yan, A., Chen, Z., 2019. Impacts of Silver Nanoparticles on Plants: A Focus on the Phytotoxicity and Underlying Mechanism. Int. J. Mol. Sci. 20, 1003. https://doi.org/10.3390/ijms20051003

Yang, X., Gondikas, A.P., Marinakos, S.M., Auffan, M., Liu, J., Hsu-Kim, H., Meyer, J.N., 2012. Mechanism of Silver Nanoparticle Toxicity Is Dependent on Dissolved Silver and Surface Coating in *Caenorhabditis elegans*. Environ. Sci. Technol. 46, 1119–1127. https://doi.org/10.1021/es202417t

Yang, Y., Guo, J., Hu, Z., 2013. Impact of nano zero valent iron (NZVI) on methanogenic activity and population dynamics in anaerobic digestion. Water Res. 47, 6790–6800. https://doi.org/10.1016/j.watres.2013.09.012

Yang, Y.-F., Cheng, Y.-H., Liao, C.-M., 2017. Nematode-based biomarkers as critical risk indicators on assessing the impact of silver nanoparticles on soil ecosystems. Ecol. Indic. 75, 340–351. https://doi.org/10.1016/j.ecolind.2016.12.051

Yasur, J., Rani, P.U., 2013. Environmental effects of nanosilver: impact on castor seed germination, seedling growth, and plant physiology. Environ. Sci. Pollut. Res. 20, 8636–8648. https://doi.org/10.1007/s11356-013-1798-3

Yu, S., Yin, Y., Liu, J., 2013. Silver nanoparticles in the environment. Env. Sci Process. Impacts 15, 78–92. https://doi.org/10.1039/C2EM30595J

Zaka, M., Abbasi, B.H., Rahman, L.-U., Shah, A., Zia, M., 2016. Synthesis and characterisation of metal nanoparticles and their effects on seed germination and seedling growth in commercially important *Eruca sativa*. IET Nanobiotechnol. 10, 134–140. https://doi.org/10.1049/iet-nbt.2015.0039

Zhai, Y., Hunting, E.R., Wouters, M., Peijnenburg, W.J.G.M., Vijver, M.G., 2016. Silver Nanoparticles, Ions, and Shape Governing Soil Microbial Functional Diversity: Nano Shapes Micro. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01123

Zhang, C., Hu, Z., Deng, B., 2016. Silver nanoparticles in aquatic environments: Physiochemical behavior and antimicrobial mechanisms. Water Res. 88, 403–427. https://doi.org/10.1016/j.watres.2015.10.025

Zhang, X., Jie, D., Liu, J., Zhang, Jianqin, Zhang, T., Zhang, Jianzhen, Ma, E., 2019. Aryl hydrocarbon receptor regulates the expression of LmGSTd7 and is associated with chlorpyrifos susceptibility in *Locusta migratoria*. Pest Manag. Sci. 75, 2916–2924. https://doi.org/10.1002/ps.5600

Zhang, Z., Song, X., Wang, Q., Lu, X., 2012. Cd and Pb Contents in Soil, Plants, and Grasshoppers along a Pollution Gradient in Huludao City, Northeast China. Biol. Trace Elem. Res. 145, 403–410. https://doi.org/10.1007/s12011-011-9199-2

Zuverza-Mena, N., Armendariz, R., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2016. Effects of Silver Nanoparticles on Radish Sprouts: Root Growth Reduction and Modifications in the Nutritional Value. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.00090