Science of the Total Environment 927 (2024) 171860

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Safe and sustainable by design Ag nanomaterials: A case study to evaluate the bio-reactivity in the environment using a soil model invertebrate

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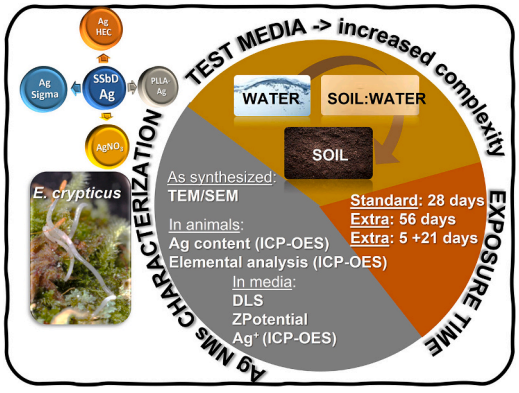
HIGHLIGHTS

- Silver nanomaterials (AgNM) levels in the environment are an increasing concern.
- Four safe-and-sustainable-by-design highly characterized AgNM forms were tested.
- Exposure done in media of increasing complexity: water, soil: water extracts and soil.
- Higher toxicity related to smaller hydrodynamic size and higher suspension stability.
- This thorough testing approach is recommended: high level of interpretation details.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
Safe and sustainability by design (SSbD)
Hazard assessment
Non-target species
Soil
Biomedical applications
Advanced materials

ABSTRACT

Safe-and-sustainable-by-design (SSbD) nanomaterials (NMs) or NM-containing products are a priority. Silver (Ag) NMs have a vast array of applications, including biomedical and other products, even as nanopesticides. Thus, their release to the environment is expected to increase. The aim of the present study was to assess the ecotoxicity of the SSbD Ag NM to the soil model species *Enchytraeus crypticus* (Oligochaeta). The Ag NM tested consists in a SSbD Ag with biomedical applications, a hydroxyethyl cellulose (HEC) coated Ag NMs (AgHEC) and its toxicity was compared to the naked Ag NMs (Ag-Sigma), an Ag-based biomedical product (PLLA-Ag: Poly L-Lactide microfibers doped with Ag), and $AgNO_3$. Effects were assessed both in soil and aqueous media, following the standard OECD guideline in soil (28 days) and the OECD extension (56 days), and short-term pulse (5 days) in aqueous media: reconstituted water (ISO water) and soil:water (S:W) extracts, followed by a 21-days recovery period in soil. Ag materials were thoroughly characterized as synthesized and during the test in media and animals. Results in S:W showed AgHEC was more toxic than Ag-Sigma (ca. 150 times) and PLLA-Ag (ca. 2.5 times), associated with a higher Ag uptake. Higher toxicity was related to a smaller hydrodynamic size and

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https://doi.org/10.1016/j.scitotenv.2024.171860
Received 25 January 2024; Received in revised form 19 March 2024; Accepted 19 March 2024
Available online 20 March 2024

higher suspension stability, which in turn resulted in a higher bioavailability of Ag NMs and released ions, particularly in S:W. Toxicity was correlated with the main physicochemical features, providing useful prediction of AgNMs bioactivity. The ability to test *E. crypticus* in a range of media with different and/or increasing complexity (water, S:W extracts, soil) provided an excellent source to interpret results and is here recommended.

1. Introduction

The European Green Deal objectives set out in the Chemicals Strategy for Sustainability (CSS) (Gottardo et al., 2021) call for a new Safe-and-Sustainable-by-Design (SSbD) approach to chemicals and emerging materials, which is aimed at developing new products that are safer, functional and more sustainable. This is part of an ambitious plan to tackle pollution from all sources and move towards a toxic-free environment (European Commission, 2020). This plan explicitly covers all Key Enabling Technology sectors, including Nanotechnology. Nanomaterials (NMs) entered the market at a faster pace than ever seen before for any other class of chemicals. In 2019, the global NM market size was valued at 8.5 billion US dollars and is expected to grow 13.1 % annually from 2020 to 2027 ("Nanomaterials Market Size, Share & Trends Analysis Report by Product (Gold, Silver, Iron, Copper), by Application (Aerospace, Automotive, Medical), by Region, and Segment Forecasts, 2021-2028," 2022). NMs offer unprecedented technological benefits as their enhanced properties at the nanoscale can produce new or improved functionalities. However, NMs also pose environmental, health and safety (EHS) concerns, which can be particularly complex with biological systems. In SSbD, all stages of the life cycle of the products, from synthesis or fabrication (e.g., avoiding the use of intrinsically toxic elements or substances, changing properties to reduce bioreactivity, etc.) till end-of-life or disposal (e.g., coating or encapsulation procedures to reduce the release of NMs from their matrix) (Cobaleda-Siles et al., 2017) should be considered, starting at the early stage of the innovation process (Hjorth et al., 2017; Kraegeloh et al., 2018; Sánchez Jiménez et al., 2020). The new aim is to go beyond simple products' safety, giving special focus to the aspect of sustainability. The current challenge – sustainability – is, among other, to ensure availability of resources and a clean environment for all, especially for future generations. The recent JRC Report: 'Safe-and-Sustainable-by-design: Framework for the definition of criteria and evaluation procedure for chemicals and materials' provides a first guide for the practical operationalization of SSbD for NMs and advanced materials (Caldeira et al., 2022). This was considered in the current study, while testing the toxicity of a Silver (Ag) NM produced within a SSbD concept, to be used in medical applications.

Nanoscale silver (Ag) has several applications, including biomedical (for instance, in drug-delivery formulations, detection and diagnosis platforms, biomaterial and medical device coatings, tissue restoration and regeneration materials (Burdușel et al., 2018)). Ag is among the most frequently used NMs, not only in consumer products but also as Ag based pesticides, that are directly applied on soils, thus is not surprising that it has already been detected in the environment (Kim et al., 2010). Among all the environmental compartments, soil is the ultimate sink for NMs, and for example a maximum predicted environmental concentration (PEC) of 45.9 mg Ag NMs/kg soil is estimated for sludge treated soils (Giese et al., 2018). Once in soils, Ag NMs can affect non-target species, such as soil invertebrates like *Enchytraeus crypticus* (Bicho et al., 2016a; Ribeiro et al., 2015; Rodrigues et al., 2020; Santos et al., 2023, 2021), *Enchytraeus albidus* (Gomes et al., 2012), *Eisenia fetida* (Baccaro et al., 2018; Courtois et al., 2021; Diez-Ortiz et al., 2015; Garcia-Velasco et al., 2016; Gomes et al., 2015b), or *Folsomia candida* (Maria et al., 2014; McKee et al., 2017; Zhang and Filser, 2020), which play key roles in the ecosystem.

Within the European Commission H2020 project BIORIMA (BIOmaterial RIsk MAnagement), alternative formulations of Ag NMs were sought by a SSbD approach. The electrospun Poly L-Lactide (PLLA) microfibers, a product manufactured for a variety of biomedical scopes, was designed and fabricated by embedding commercial Ag-Sigma [Sigma-Aldrich] into the fibres for antimicrobial wound dressing applications (further referred to as PLLA-Ag). The SSbD Ag material (produced within BIORIMA project) consists of an alternative biocompatible coating based on hydroxyethyl cellulose (HEC) surrounding Ag NMs (further referred to as AgHEC). The synthesis of AgHEC, patented by Costa and Blosi (2016), is an affordable and eco-friendly process, entirely carried out at room temperature in presence of benign reagents and water based – economically and environmentally sustainable. The HEC coating is a benign polymer widely used in cosmetics and with known low toxicity and environmental impact, hence reduced hazard is expected. Also, being positively charged and bearing a quaternary ammonium group, HEC is reported to be highly active in preventing Gram (−) bacteria infection and resistance (Alfei and Schito, 2020; Jain et al., 2014; Kenawy et al., 2007). AgHEC has shown to be a good alternative to chloroquine, considering their risk/benefit profile, for use as antimicrobial (against *Escherichia coli*) and antiviral (against SARS-COV-2) agent in solution, as fabric coating and embedded in hydrogel scaffolds (Costa et al., 2022). Further, AgHEC showed enhanced antimicrobial activity against pathogenic strains compared to commercial Ag NMs (Marassi et al., 2018), i.e. improved product performance. However, a recent study showed that, based on a Adverse Outcome Pathway analysis for lung fibrosis, AgHEC are likely more hazardous than naked- and PVP- coated-Ag NM (Motta et al., 2023).

The environmental effects of AgHEC are currently unknown, and its safety should be assessed. Thus, the aim of the present study was to assess the toxicity/bioactivity of AgHEC (the proposed SSbD material), in comparison to Ag-Sigma, PLLA-Ag and $AgNO_3$, using the non-target soil invertebrate *E. crypticus* (Oligochaeta) model. Enchytraeids are standard model species in soil ecotoxicology, with standard guidelines to assess effects on survival and reproduction (ISO 16387, 2023; OECD 220, 2016). They have a widespread distribution and occur in large numbers in most soils, where they play an important role in ecological functions like organic matter decomposition and soil bioturbation, improving the small-scale water and air management of soil (Didden and Rombke, 2001; Jansch et al., 2005). They live in the interstitial soil-pore water, and a short-term aquatic toxicity test system has been proposed for *E. albidus* (Rombke and Knacker, 1989) and further adapted (Gomes et al., 2015a) and replicated in *E. crypticus* (Bicho et al., 2016b; Gomes et al., 2018b; Rodrigues et al., 2020). This test system allows the testing of NMs using a less complex test media (reconstituted water) compared to soil. The toxicity assessment was done both in soil and in aqueous media, the latter followed by post-exposure in clean soil to assess effects, as in Gomes et al. (2015a). We hypothesize that the increase in matrix complexity: from water, via soil:water extracts, to soil, can help discriminate toxicity factors and enhance the interpretation. This, linked to the thorough characterisation performed in all media will deliver a most up to date and complete dataset, which is extremely limited when considering the complex soil matrix.

2. Materials and methods

2.1. Test species

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. The cultures were kept in agar, consisting of sterilized Bacti-Agar medium (Oxoid, Agar No. 1) and a mixture of four different salt solutions at the final concentrations of 2 mM $CaCl_2{\cdot}2H2O$, 1 mM $MgSO_4$,

0.08 mM KCl, and 0.75 mM $NaHCO_3$, under controlled conditions of temperature (19 ± 1 °C) and photoperiod (16:8 h light:dark). The cultures were fed with ground autoclaved oats twice per week.

2.2. Test materials

Silver nitrate ($AgNO_3$, Sigma-Aldrich, ≥99 % ACS reagent) and three Ag based nanomaterials: Ag nanopowder (Ag-Sigma) (<150 nm, 99 %, Sigma-Aldrich), hydroxyethyl cellulose coated Ag (AgHEC) (10 % Ag, 90 % cellulose), and Poly L-lactide nanofiber with embedded Ag nanoparticles (PLLA-Ag) (5 % Ag), were tested.

Ag-Sigma was used as purchased. AgHEC is a solid fibrous powder prepared by spray freeze drying the water-based AgHEC suspension (0.1 % wt) obtained by means of a patented eco-friendly procedure (Costa and Blosi, 2016) which exploits the cationic quaternized hydroxyethylcellulose both as chelating and as reducing agent. PLLA-Ag is a material designed for wound dressing applications and composed by electrospun Poly L-Lactide (PLLA) microfibers doped with Ag NMs [the fibres mean diameter is ~4 μm with a thickness of the electrospun layer of 50 μm]. PLLA-Ag were grinded by a high shear process, consisting of a series of steps to obtain a homogeneous fibre suspension, as follows: i) immersion of the fibre sample in ethanol and cooling in liquid nitrogen, ii) knife mill for 2 min and drying at 60 °C, iii) incorporation of the solid in water (8.75 mg of fibre sample and 30 g of distilled water) and treatment through a high shear turbine (Ultraturrax) for 2 min at 17,000 rpm. The so obtained suspension was let to settle down and separate in two phases; the top (deflocculated) phase corresponds to the sample PLLA-Ag that was stored at −80 °C until use. For the tests, the sample was let to defrost and manually shaken for 2 min.

2.3. Test media and spiking

2.3.1. Soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics are pH (0.01 M $CaCl_2$) of 5.5, 1.77 %, organic matter, 10.1 meq/100 g CEC (cation exchange capacity), 44.8 % WHC (water holding capacity), 7.3 % clay, 13.8 % silt, and 78.9 % sand regarding grain size distribution.

The tested concentrations were 0, 32, 100, 320, 1000, and 3200 mg Ag/kg soil for Ag-Sigma, 0, 32, 100, 320, and 1000 mg Ag/kg soil for AgHEC, and 0, 10, 30, and 60 mg PLLA-Ag/kg soil for PLLA-Ag, corresponding to 0, 0.5, 1.5, and 3 mg Ag/kg soil.

Ag-Sigma and AgHEC were directly mixed with the dried soil, following the recommended method for dry powder nondispersible nanomaterials (OECD, 2012), done per individual replicate to ensure total raw amounts per replicate, except for 32 mg Ag/kg soil of Ag-Sigma which was prepared in a batch of 2 replicates to ensure weight precision. To note that AgHEC has a fibrous aspect (see Fig. S1, Supplementary material) that seem to not completely mix with soil.

For PLLA-Ag, the stock (aqueous) suspension was serially diluted and added to the pre-moistened soil, each replicate prepared individually, and the soil was homogeneously mixed.

$AgNO_3$ was added to pre-moistened soil batches (per concentration) as serially diluted aqueous solutions as previously described (Bicho et al., 2016a). Soils' moisture was adjusted to 50 % of the soil's maxWHC adding deionised water. The soil was left to equilibrate for 24 h prior the start of the tests.

The effects in terms of survival and reproduction for $AgNO_3$ were already known by the authors (Bicho et al., 2016a), thus this soil test was not repeated here.

2.3.2. ISO water

Reconstituted standard International Organization for Standardization (ISO) water was used (OECD 202, 2004), being composed of 2 mM of $CaCl_2.2H_2O$, 0.5 mM of $MgSO_4.7H_2O$, 0.77 mM of $NaHCO_3$, and 0.077 mM of KCl in ultrapure water.

Test concentrations were 0, 0.5, 1, 5, 10 mg Ag/L for $AgNO_3$, 0, 1, 10, and 100 mg Ag/L for Ag-Sigma and AgHEC, and 0, 1, 10, and 100 mg PLLA-Ag/L for PLLA-Ag, equivalent to 0, 0.05, 0.5 and 5 mg Ag/L. The suspensions were prepared at twice the concentrations tested (e.g., at 2 mg/L, 20 mg/L, and 200 mg Ag/L) using ultrapure water and hand shaken for 1–2 min. After mixing, 0.5 mL of the suspensions were immediately added to each well of the test plates containing 0.5 mL of the test medium (ISO water). The animals' exposure started immediately after.

2.3.3. Soil:water extracts

Soil:water extracts were obtained by mixing LUFA 2.2 soil and ultrapure water in a proportion of 1:5 (w/v) under an orbital shaker for 5 min, at 250 rpm. After that, the mixture was let to settle for 2 h. The supernatant was collected and filtered through a 50 μm glass microfibre filter to avoid larger surface material. The soil:water extract was stored at 4 °C until use (2 days maximum). Test concentrations and spiking followed the same as for ISO water.

2.4. Exposure procedures

Toxicity tests were performed in three different test media, with different characteristics and complexity. The following sections will describe the test procedures separately: 1) soil tests, which include the standard test procedures and its extension for a longer-term exposure test, 2) aqueous exposures, which include ISO water and soil:water extracts tests, and 3) post-exposure in clean soil which followed the aqueous exposures, i.e., using the surviving animals.

2.4.1. Soil tests (standard OECD and standard OECD extension)

The standard guideline for the Enchytraeid Reproduction Test (ERT) (OECD 220, 2016) was followed, 28 days exposure, plus an extension as described in Ribeiro et al. (2018), i.e. 56 days, and including extra sampling times, 7, 14, and 21 days. Endpoints include survival in all the sampling times, reproduction at days 28 and 56, and size measurement, i.e. impact on growth, at day 28. One replicate was performed for the days 7, 14 and 21, and four replicates, for the days 28 and 56. Briefly, 10 synchronized age animals were introduced in each test container with moist soil (⌀4 cm with 20 g of soil for days 7, 14, 21, and 28, and ⌀5.5 cm with 40 g of soil for day 56) and food supply (24 ± 2 mg, autoclaved rolled oats). Test ran during 56 days at 20 ± 1 °C and 16:8 h photoperiod. Food (12 ± 1 mg: until day 28, and 24 ± 2 mg: from 28 to 56 days) and water were replenished every week. At days 7, 14 and 21, one replicate of each treatment was monitored for survival. At day 28, survival and reproduction were assessed by counting the juvenile and adult organisms, which were fixed with ethanol and stained with Bengal rose (1 % in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile animals were counted using a stereo microscope and survival and reproduction assessed. Adult animals were photographed for posterior determination of size (length) using the software Image J (Schneider et al., 2012). For the 56 days' exposure replicates, adults were carefully removed from the soil at day 28, after which the exposure continued until day 56, when the animals were counted, following the same procedure, as described for the day 28.

The animals sampled at days 7, 14, 21, and 28 were carefully washed and depurated in ISO water for 24 h, snap frozen and stored at −80 °C until further analysis.

2.4.2. Alternative water tests (ISO water and soil:water extracts)

Test procedures followed the same as described in Gomes et al. (2015a) based on the initial concept developed by Rombke and Knacker (1989), and replicated since in several studies (Bicho et al., 2016b; Gomes et al., 2018b; Rodrigues et al., 2020). The endpoint was survival, monitored every 24 h. For each test condition, five adult animals with

well-developed clitellum and similar size were selected per replicate. Ten replicates per treatment were used. The exposure was performed in 24-well plates, where each well corresponds to one replicate and contained 1 mL of the corresponding test solution. The test duration was 5 days, at 20 ± 1 °C and 16:8-h photoperiod.

The surviving animals were collected at the end of the exposure (day 5), 40 out of 50 were transferred to clean soil (see below) and the remaining 10 were snap frozen and stored at −80 °C until further analysis.

2.4.3. Post-exposure in clean soil (after aqueous exposure)

The animals exposed via ISO water and soil:water were transferred to control (non-spiked) LUFA 2.2 soil, as described in Gomes et al. (2015a). Endpoints include survival and reproduction. The procedure followed the OECD standard guideline i.e., 21 days' duration. In short, the surviving animals from each test condition were pooled in groups of 10 and introduced on test vessels with soil. Four replicates per pre-exposure condition were performed. At the test end, survival and reproduction were assessed by counting the juvenile and adult organisms, as described for soil tests.

2.4.4. Data analysis

To assess differences between treatments and controls (for each exposure period), one-way analysis of variance (ANOVA) was performed, followed by the post hoc Dunnett's method for multiple comparisons, at a significance level of 0.05 (SigmaPlot 11.0).

Effect concentrations (ECx) were calculated modelling data to logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 1 using the Toxicity Relationship Analysis Program (TRAP 1.30) software.

2.5. Materials characterisation

2.5.1. As synthesized

Electron transmission microscopy (TEM) analyses were performed by means of a FEI TECNAI F20 instrument microscope operating at 200 keV. Ag suspensions were drop-casted on a holey carbon film supported by a gold grid. The specimen was then dried at 60 °C. To gather information about particles morphology the images were taken in phase contrast mode and high-angle annular dark-field scanning transmission mode (HAADF-STEM). The grids were analyzed by TEM and evaluated using an automated image analysis (ImageJ Software V1.53). AgHEC in powder after spray freeze drying treatment was observed by scanning electronic microscopy analysis using a Field Emission Scanning Electron Microscope, FE-SEM (Carl Zeiss Sigma NTS, Germany).

2.5.2. In test media

2.5.2.1. Preparation of stock suspensions and dilution in test media. Ag-Sigma and AgHEC suspensions were prepared in MilliQ (MQ) water at concentration of 2000 mg/L considering the NMs purity. For example, to prepare 20 mL of AgHEC suspension 400 mg of AgHEC (10%wt Ag and 90%wt HEC) were added to 20 mL of MQ water. The suspensions were vortexed for 2 min until no precipitates were observed. All the prepared stock suspensions (2000 mg/L) were diluted in media (ISO water and soil water extract) at 10, 100 and 1000 mg/L. PLLA-Ag fibres were diluted from the stock solution in both media at a concentration of 2 mg/L and incubated in static conditions at 20 °C. In alignment with the in-vivo conditions, all the prepared suspensions, including $AgNO_3$ solutions at the same concentrations, were incubated in static conditions at 20 °C for 1, 5, 28 and 56 days.

2.5.2.2. Colloidal behaviour. Hydrodynamic diameter (d_DLS) and Zeta Potential (ZP) were determined for the nanomaterials dispersed in ISO water and in soil:water extracts at the tested concentrations and incubation times by means of Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) techniques with a Zetasizer Nano instrument ZSP (model ZEN5600, Malvern Instruments, UK). Smoluchowski equation was applied to convert the electrophoretic mobility to ZP. Samples were measured three times and d_DLS and ZP data with relative standard deviations (rsd %) were obtained by averaging three independent measurements.

2.5.2.3. Static dissolution. Static dissolution measurements were made to assess the release of Ag^+ from Ag NPs. Samples prepared at different exposure conditions were filtered through 10 kDa molecular weight cut-off membranes (4200 rpm for 45 min) and the filtrated solution analyzed by ICP-OES. Elemental analysis was performed by an ICP-OES 5100 vertical dual view apparatus (Agilent Technologies, Santa Clara, CA, USA). The analysis was performed in radial viewing mode, and calibration curves were obtained with 0.05, 0.1, 1, 10, 100 and 1000 mg/L standards for Ag element. Nitric acid was added both to standards and to diluted samples (1:10 *v*/v). The concentration of Ag^+ in washing water was directly evaluated by ICP-OES determination. Calibration curve was evaluated and showed a good correlation, coefficient (R^2) above 0.99. Results from ICP-OES were reported as the average of three independent measurements with relative standard deviation (RSD) %.

2.5.3. In the animals: elemental quantification analysis

The sampled stored animals were freeze-dried for 48 h, weighted, and transferred to glass vials. Each sample was treated with 150 μL of H_2O_2 (30 % vol) and 150 μL of HNO_3 (65 % vol). The samples were kept under magnetic stirring at 500 rpm over a heating plate at 40 °C for 8 h, then cooled overnight. After that, 1.2 mL of MilliQ water were added into the solution. Elemental analysis was performed by an ICP-OES 5100 vertical dual view apparatus (Agilent Technologies, Santa Clara, CA, USA). The analysis was carried out in radial viewing mode, and calibration curves were obtained with 0.01, 0.05, 0.1, 1, 10, 100 and 1000 mg/L standards for Ag and also additional elements: Cu, Fe, Mg, Ni and Zn. HNO_3 and H_2O_2 were added both to standards and to diluted samples (1:10 v/v). Calibration curve was evaluated and showed a good correlation, coefficient (R^2) above 0.99. Results from ICP-OES were reported as the average of three independent measurements.

3. Results

3.1. Soil tests (standard OECD and standard OECD extension)

The validity criteria were fulfilled as in the standard OECD test, i.e., in controls, adult mortality <20 % and the number of juveniles >50, with a coefficient of variation <50 %.

Results from the exposure via soil (Fig. 1) showed that for Ag-Sigma and AgHEC the effect increased in the standard OECD extension (56 days) compared to the standard OECD (28 days). PLLA-Ag was not toxic, but the tested range was not comparable (due to technical reasons, the *as synthesized* PLLA-Ag suspension has a maximum Ag content of 5 %), being much lower than for Ag-Sigma and AgHEC.

Ag-Sigma induced dose-dependent effects after 28 days of exposure (survival: LC50 = 1276 mg Ag/kg soil; reproduction: EC50 = 446 mg Ag/kg soil) and after 56 days (EC50 = 500 mg Ag/kg soil). AgHEC (28 days of exposure) reduced reproduction in a dose-dependent way (EC50 = 686 mg Ag/kg soil) but without effects on survival up to 1000 mg/kg; after 56 days effects were comparatively higher (EC50 = 448 mg Ag/kg soil). PLLA-Ag did not affect survival or reproduction up to 60 mg PLLA-Ag/kg soil (corresponding to 3 mg Ag/kg soil), either after 28 or 56 days of exposure. $AgNO_3$ was the most toxic (ERT LC50 and EC50 = 62 and 75 mg Ag/kg soil) (Bicho et al., 2016a).

No differences were observed in terms of size of the adults at day 28 (see Supporting information, Fig. S2). Estimated effect concentrations for all tests are presented on Table 1.

Table 1
Effect concentrations (ECx) estimated for survival (Surv) and reproduction (Rep) of *Enchytraeus crypticus* when exposed to Ag nanopowder (Ag-Sigma), hydroxyethyl cellulose coated Ag NPs (AgHEC), Poly L-lactide nanofiber with embedded Ag NPs (PLLA-Ag), and $AgNO_3$ in LUFA 2.2 soil, ISO reconstituted water (ISO) and soil:water extracts (S:W). Results are expressed as mg Ag/kg soil for soil tests; and mg Ag/L for ISO water, S:W extracts and post-exposure tests [in the Table as soil (ISO) and soil (S:W)]. The 95 % confidence intervals (CI) are shown in brackets. The models used were Logistic 2 parameters (Log2P) or Threshold sigmoid 2 parameters (Thres2P). S: slope; Y0: intercept; n.d.: not determined; n.e.: no effect. *from Bicho et al. (2016a); # there were no animals transferred to soil, all died during exposure in S:W, LOEC (Lowest Observed Effect Concentration) = 0.05 mg Ag/L.

End-point	Test media	Time (days)	EC10 (95 % CI)	EC50 (95 % CI)	EC90 (95 % CI)	Model & parameters
Ag-Sigma						
Surv	Soil	28	169 (−344–683)	1276 (749–1803)	2383 (1087–3678)	Log2P S:4.9E-4; Y0:9.8
Rep	Soil	28	n.d.	446 (235–659)	742 (155–1328)	Thres2P S:1.2E-3; Y0:935
Rep	Soil	56	n.d.	500 (302–697)	816 (306–1325)	Thres2P S:1.1E-3; Y0:4297
Surv	ISO	5	87 (39–135)	220 (28–412)	353 (−19–725)	Log2P S:4.1E-3; Y0:5
Surv	Soil (ISO)	21	109 (36–183)	255 (−204–715)	345 (−369–1060)	Thres2P S:4.9E-3; Y0:9.8
Rep	Soil (ISO)	21	n.e.	n.e.	n.e.	
Surv	S:W	5	122 (24–221)	299 (−171–770)	409 (−303−1120)	Thres2P S:3.1E-3; Y0:4.7
Surv	Soil (S:W)	21	110 (22–197)	166 (−367–699)	223 (−762–1208)	Log2P S:9.7E-3; Y0:9.4
Rep	Soil (S:W)	21	86 (−4218–4391)	108 (−2394–2610)	129 (−9179–9437)	Log2P S:2.6E-2; Y0:189
Ag-HEC						
Surv	Soil	28	804 (476–1132)	1614 (693–2535)	2424 (463–4385)	Log2P S:6.8E-4; Y0:9.9
Rep	Soil	28	81 (−181–344)	686 (501–872)	1291 (923–1660)	Log2P S:9.1E-4; Y0:953
Rep	Soil	56	69 (−125–262)	448 (253–643)	828 (365–1290)	Log2P, S:1.5E-3; Y0:4371
Surv	ISO	5	n.e.	n.e.	n.e.	–
Surv	Soil (ISO)	21	n.e.	n.e.	n.e.	–
Rep	Soil (ISO)	21	n.e.	n.e.	n.e.	–
Surv	S:W	5	0.8 (0.1–1.4)	1.9 (−0.4–4.3)	3.1 (−2.1–8.2)	Log2P S:4.7E-1; Y0:4.1
Surv	Soil (S:W)	21	5.8 (−)	6.6 (−)	7.5 (−)	Log2P S:6.5E-1; Y0:9.5
Rep	Soil (S:W)	21	6.1 (−)	6.9 (−)	7.7 (−)	Log2P S:6.5E-1; Y0:125
PLLA-Ag						
Surv	Soil	28	n.e.	n.e.	n.e.	–
Rep	Soil	28	n.e.	n.e.	n.e.	–
Rep	Soil	56	n.e.	n.e.	n.e.	–
Surv	ISO	5	n.e.	n.e.	n.e.	–
Surv	Soil (ISO)	21	n.e.	n.e.	n.e.	–
Rep	Soil (ISO)	21	3.2 (−0.7–7.1)	11.2 (−2.6–24.9)	19.1 (−8.6–46.9)	Log2P S:6.9E-2; Y0:532
Surv	S:W	5	4.1 (−1668–1676)	4.9 (−166–175)	5.7 (−1326–1337)	Log2P S:6.9E-1; Y0:4.6
Surv	Soil (S:W)	21	2.2 (0.3–4.0)	5.8 (4.1–7.5)	9.5 (5.6–13.4)	Log2P S:1.5E-1; Y0:9.6
Rep	Soil (S:W)	21	0.6 (−2.4–3.6)	5.4 (1.6–9.2)	10.2 (2.0–18.3)	Log2P S:1.2E-1; Y0:124
$AgNO_3$						
Surv	Soil	21	52 (38–67)*	75 (66–84)*		Log2P S:2E-2; Y0:9.2
Rep	Soil	21	38 (24–51)*	62 (57–68)*		Log2P S:2E-2; Y0:390
Surv	ISO	5	4.2 (0.1–8.3)	13.8 (8.6–19.0)	23.5 (10.9–36.0)	Log2P S:5.7E-2; Y0:5
Surv	Soil (ISO)	21	n.e.	n.e.	n.e.	–
Rep	Soil (ISO)	21	n.e.	n.e.	n.e.	–
Surv	S:W	5	n.d. (<0.05)	n.d. (<0.05)	n.d. (<0.05)	–
Surv	Soil (S:W)	21	n.d. #	n.d.#	n.d.#	–
Rep	Soil (S:W)	21	n.d.#	n.d.#	n.d.#	–

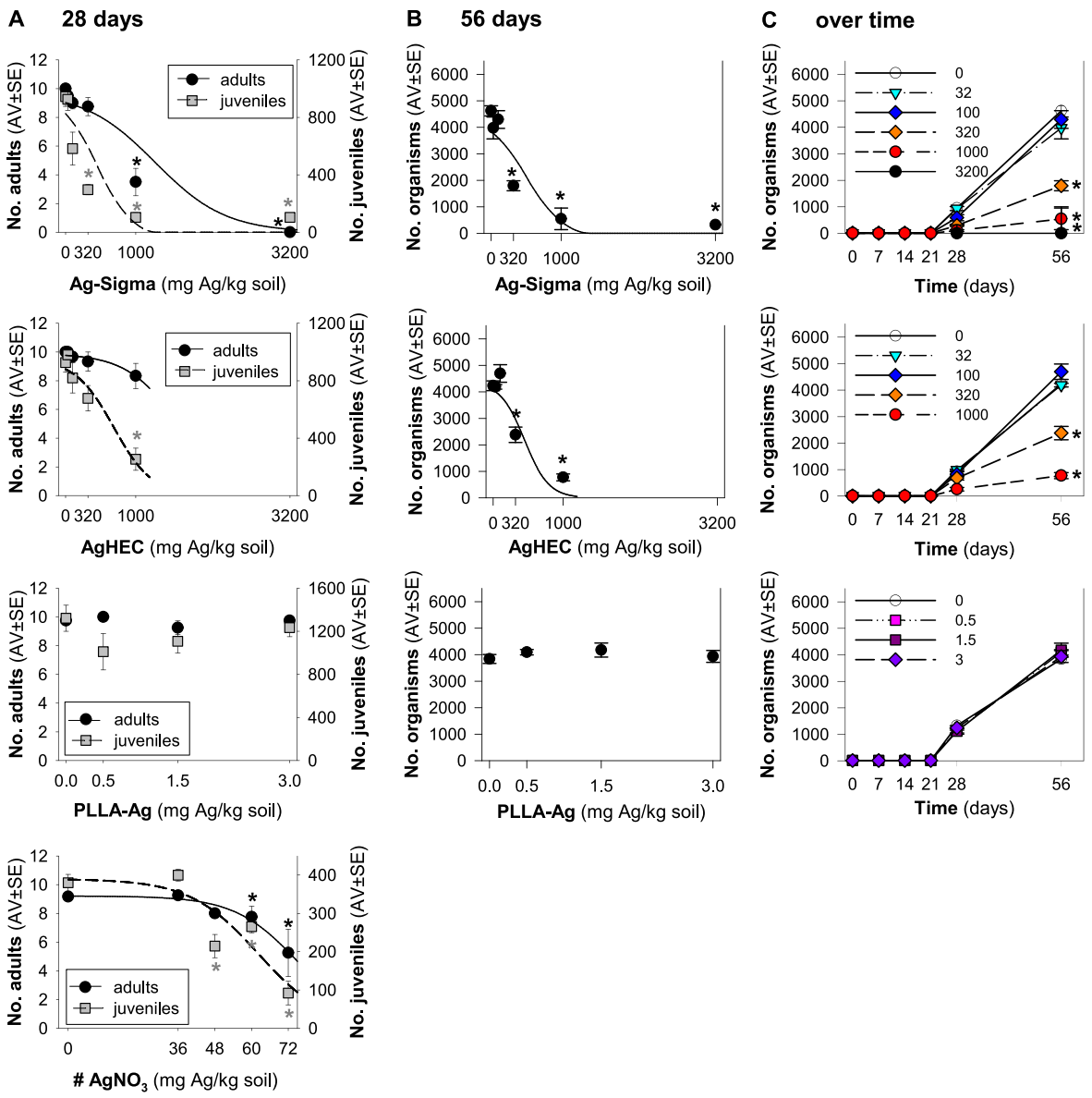

Fig. 1. Survival and reproduction of *Enchytraeus crypticus* when exposed to Ag nanopowder (Ag-Sigma), hydroxyethyl cellulose coated Ag NPs (AgHEC), and Poly L-lactide nanofiber with embedded Ag NPs (PLLA-Ag) in LUFA 2.2 soil during A) 28 days (Standard OECD Enchytraeid Reproduction Test (ERT)) and the OECD extension B) 56 days, and C) over time series sampling at days: 7, 14, 21, 28 and 56 days. The lines in A and B represent the model fit to data. Values represent number of adults, juveniles, and population as average ± standard error (AV ± SE). *: $p < 0.05$ (Dunnett's method). # data from (Bicho et al., 2016a).

3.2. Alternative water tests (ISO water and soil:water extracts) and post-exposure in clean soil (after aqueous exposure)

Results from the exposure via ISO water and post-exposure in clean soil (Fig. 2) showed higher toxicity of $AgNO_3$ followed by AgHEC, with non-monotonic responses (higher effects at intermediate concentrations). The organisms recovered after transfer to clean soil.

Ag-Sigma (up to 100 mg Ag/L) and PLLA-Ag (up to 5 mg Ag/L) caused no significant acute effects via ISO water exposure for 5 days, nor when exposed animals were transferred to clean soil for 21 days. AgHEC induced significant mortality at 10 mg Ag/L (but not at 100 mg Ag/L) after 5 days of exposure via ISO water, however the animals recovered when transferred to clean soil (no effects on survival or reproduction). $AgNO_3$ induced mortality, significantly at the intermediate concentration 5 mg Ag/L (but not at 10 mg Ag/L), after 3 days of exposure via ISO water, but again animals (survivors) recovered when transferred to clean soil for 21 days.

Results from the exposure via soil:water extracts and post-exposure in clean soil (Fig. 3) showed overall higher toxicity than observed in ISO water, for the same materials.

Ag-Sigma (up to 100 mg Ag/L) caused no significant acute effects via S:W extracts exposure, however, when transferred to clean soil, the animals pre-exposed to 100 mg Ag/L in S:W extracts have a significantly lower reproductive output after 21 days. All the animals exposed to 100 and 10 mg Ag/L of AgHEC via S:W extracts were dead, after 24 h and 48 h of exposure, respectively; exposure to 1 mg Ag/L caused no acute toxicity (5 days exposure in S:W extracts) nor after 21 days post-exposure in clean soil. PLLA-Ag induced acute toxicity, at the concentration of 5 mg Ag/L, after the 3rd day of exposure in S:W extracts; the toxicity persisted after 21 days of post-exposure in clean soil, with reduced survival and reproduction, at similar effect concentrations (EC50 of 4.9, 5.8, and 5.4 mg Ag/L for survival in S:W extracts, survival in post-exposure and reproduction in post-exposure, respectively). $AgNO_3$ caused 100 % mortality in all the concentrations tested (after 24 h for 0.5, 5, and 10 mg Ag/L, and after 48 h for 0.05 mg Ag/L); thus, there were no animals to transfer to clean soil.

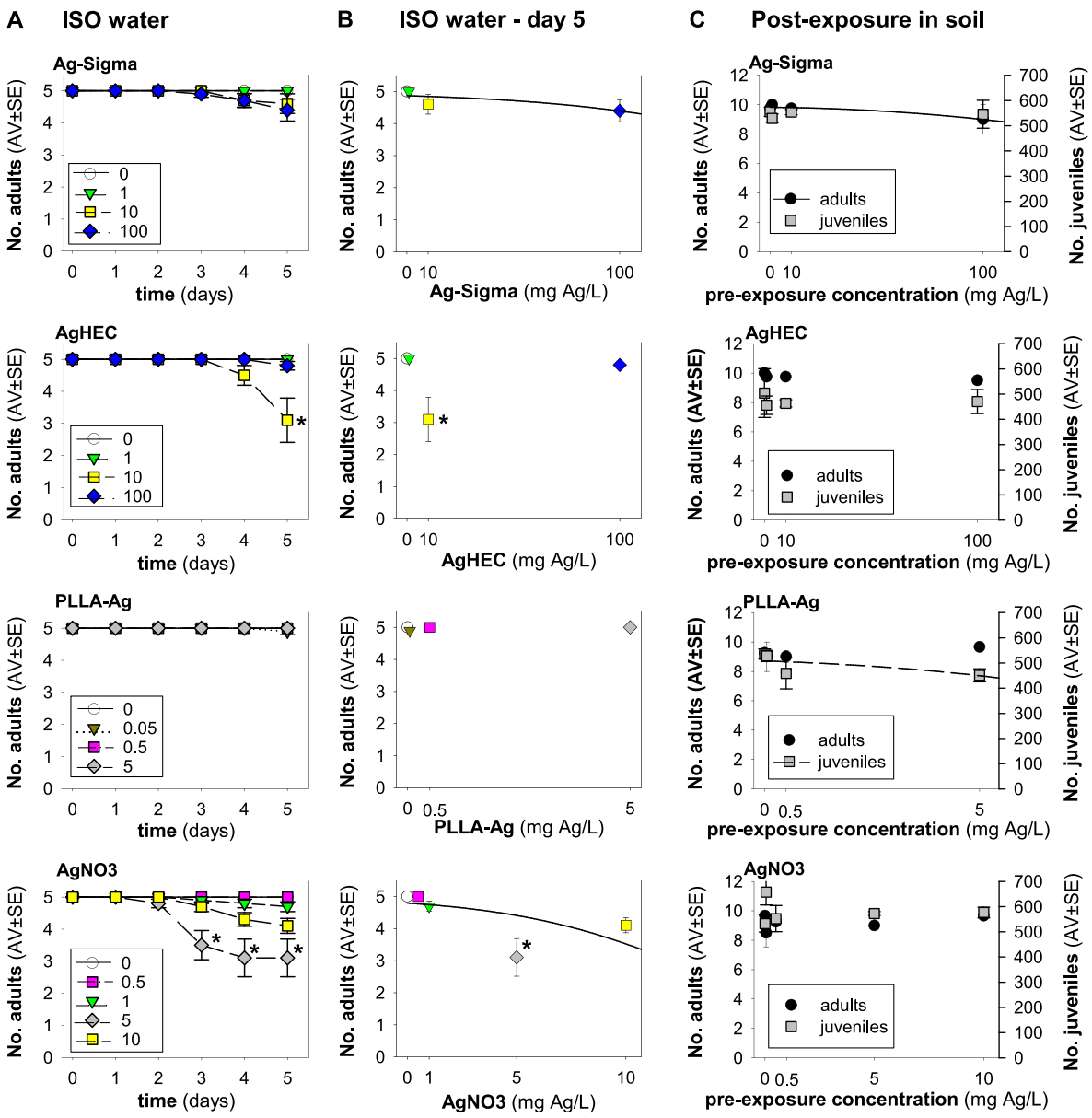

Fig. 2. Survival and reproduction of *Enchytraeus crypticus* when exposed to Ag nanopowder (Ag-Sigma), hydroxyethyl cellulose coated Ag NPs (AgHEC), Poly L-lactide nanofiber with embedded Ag NPs (PLLA-Ag), and $AgNO_3$ (mg Ag/L). A) Exposure in ISO water media from 1 to 5 days. B) Exposure in ISO water for 5 days. The concentration is expressed as mg Ag/L. C) Post exposure in clean LUFA 2.2 soil, for 21 days, after 5 days in ISO water media. All values are expressed as average ± standard error (Av ± SE). *: $p < 0.05$ (Dunnet's method). The lines in B and C represent the model fit to data.

Visual inspection of the exposure (Figs. S3 and S4) showed the precipitation of the NMs forming aggregates in the bottom on the well-plates, independently of the test media (ISO water or S:W extracts) and the adherence of the NMs to the animals' dermis, particularly in the clitellum region.

3.3. Materials characterisation

3.3.1. As synthesized

TEM images collected by HAADF-STEM mode (Fig. S5) showed for Ag-Sigma crystalline particles with irregular morphology and size ranging from 10 to 150 nm as consistent with the high temperature processes applied for their production. AgHEC images highlighted spherical regular nanoparticles morphology with diameter ranging from 3 to 20 nm with a mean diameter of 9 ± 1 nm measured on >150 particles.

The *as synthesized* PLLA-Ag sample in water (after the high shear process, see Section 2.2 for details) presents micrometric hydrodynamic diameter in the range of ca. 1000 μm associate with high polydispersity index (Table S1). Zeta-potential value was highly negative and consistent with a good colloidal stability.

3.3.2. In test media

Visual inspection/observation of the samples showed that Ag-Sigma presented a high instability in both aqueous media (ISO water and S:W extracts), precipitating in few minutes. The pH was not affected by concentration of Ag-Sigma or exposure time and were stable at ca. pH 7 in both media. Size distribution (based on DLS) were in the micrometric range for all concentrations and exposure times, indicating a strong aggregation of the NPs (Tables S2 and S3. The PDI were high (>0.4) in almost all exposure conditions, indicating the presence of poly-dispersed aggregates. In ISO water, size distribution decreased with increasing concentrations, and the effects of time were not linear (higher PDI at day 5). In S:W extracts, the size distribution was lower than in ISO water,

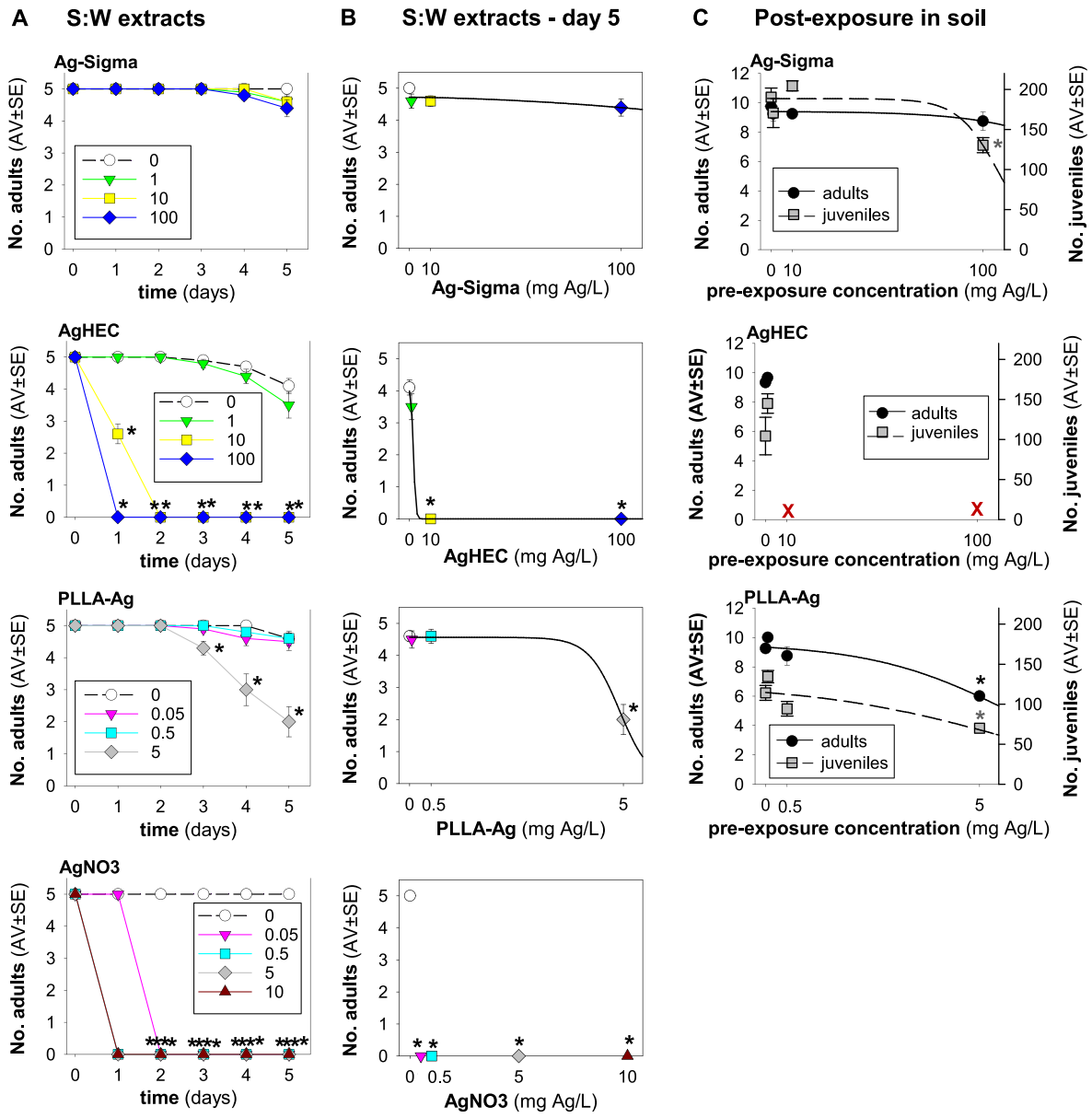

Fig. 3. Survival and reproduction of *Enchytraeus crypticus* when exposed to Ag nanopowder (Ag-Sigma), hydroxyethyl cellulose coated Ag NPs (AgHEC), and Poly L-lactide nanofiber with embedded Ag NPs (PLLA-Ag), and $AgNO_3$ (mg Ag/L). A) Exposure in soil:water (S:W) extracts media from 1 to 5 days. B) Exposure in S:W extracts for 5 days. C) Post exposure in clean LUFA 2.2 soil, for 21 days, after 5 days in ISO water media. All values are expressed as average ± standard error (Av ± SE). *: $p < 0.05$ (Dunnet's method). X: no animals transferred from S:W exposure. The lines in B and C represent the model fit to data.

with similar trend of decreasing size with increasing concentrations; here, the smaller sizes were reported at day 1. All these trends are explained by the precipitation of larger agglomerates not detectable by DLS, whose formation is promoted by increase of the Ag NM concentration. The presence of negative charged organic compounds in S:W extracts could improve dispersibility, ensuring negative zeta-potential stably kept (ca. -20 mV) for all the concentrations. The colloidal equilibrium between NPs and the medium probably occurred within the first 24 h and is preserved during the 56 days of exposure. Otherwise in ISO water, the progressive shift of zeta-potential to more negative values with time is due to a progressive agglomeration of particles that can exchange the surface acidity, promoting the stabilisation of more negative charge (Costa et al., 2013).

AgHEC was well dispersed, producing homogeneous suspensions in both media. The high pH of the AgHEC stock suspension (pH 12 at 2000 mg/L in MilliQ water) resulted in basic pH after adding to the test media (ISO water and S:W extracts), that increased with increasing Ag concentrations (Tables S4 and S5). Further, pH did not change significantly with time, for both ISO water and S:W extracts (Tables S4 and S5). The size distribution of AgHEC in ISO water is monomodal, with narrow and very resolved peaks and size diameters stable as a function of time. The increasing of DLS size at the higher concentrations, is due to the expected progressive agglomeration of particles, still remaining dispersed in the colloidal range. The slight tendency for agglomeration as a function of concentration, is also confirmed by PDI values, from ca. 0.25 for 10 mg/L samples to 0.35 for 1000 mg/L samples. In S:W extracts, the hydrodynamic diameter and zeta-potential values were comparable to samples in ISO water for the concentrations 100 and 1000 mg/L. However, for the 10 mg/L samples, the DLS measurements reported micrometric diameter and high PDI values, suggesting that, at such low AgHEC concentration, the DLS technique mainly detects the medium suspended salts, overlapping Ag signal. As expected, zeta-potentials confirmed a positive surface charge in both media due to the adsorption of cationic quaternized hydroxyethylcellulose (HEC) on

AgNPs, that due to the high surface to volume area is not neutralised by negatively charged organic compounds.

For $AgNO_3$ samples, the formation of precipitates, most likely attributed to AgCl, Ag_2CO_3, and Ag_2SO_4 species, was observed, both in ISO water and S:W extracts, in the form of grey sediment at the bottom of the vials. The observation of the precipitates was confirmed by DLS results (Tables S6 and S7). In both media large aggregates of micrometric size and broad dispersity were detected, presenting the same variability of Ag-Sigma NPs where the decrease of DLS size over time and at increasing concentration is due to the separation of largest agglomerates by the medium.

For PLLA-Ag, dispersion in ISO water (Table S8) did not cause significant changes in DLS size over time, preserving high polydispersity and negative zeta potentials throughout the overall exposure time. In S:W extracts (Table S9) the results pointed out a slight decrease in mean size and PDI values, in line with the hypothesized dispersion effect promoted by the organic compounds in medium and already observed for Ag-Sigma. Negative and stable zeta-potentials were observed in this medium as well.

Ag concentration measured in aqueous media (Fig. 4) showed different patterns, as based on the Ag content measured in the media (dissolution), between Ag materials, and ISO water versus S:W extracts.

In ISO water both Ag-Sigma and AgHEC showed evidence of low dissolution, below 0.04 mg/L, and a scarce dependency from the initial concentration and the exposure time, typical behaviour indicating the achievement of a dissolution equilibrium in this medium. Only a slight dissolution increase was observed from day 4 to 28. The dissolution of AgHEC in S:W was slightly higher, in the range of 0.01–0.2 mg/L, but similar for all the tested concentrations, with a slight increase from 4 to 28 days. These findings, for all the concentrations, can be associated with AgCl, Ag_2CO_3, and Ag_2SO_4 forming a solid layer on the surface of the nanoparticles that prevent the dissolution process, due to Cl^-, CO_3^{2-} and SO_4^{2-} presence in ISO water and S:W extracts. Ag-Sigma in S:W showed different dissolution profiles for the three Ag concentrations and consistent with the presence of three solid/liquid equilibria, most likely due to the action of complexing agents (Cl^-, CO_3^{2-} and SO_4^{2-}). Overall, the percentage dissolution detected for both Ag-Sigma and AgHEC, in all the tested conditions was lower than 1 %.

Ion detection of $AgNO_3$ dispersed in the media provided some information on the role of Cl^-, CO_3^{2-} and SO_4^{2-} in the dissolution equilibria (see Fig. 4). In ISO water, the Ag^+ content measured for $AgNO_3$ was similar to Ag-Sigma and AgHEC at 10 and 100 mg/L, in line with an almost total subtraction of Ag^+ from the solution, while for 1000 mg/L, almost 60 % of Ag still remains in solution. In S:W extracts, the anions action was lower and the precipitation occurred only for the concentration 10 mg/L, while at 100 mg/L and 1000 mg/L the Ag detected in solution were 60 % and 100 %, respectively. This finding suggests that the Cl^-, CO_3^{2-} and SO_4^{2-} species present in the ISO and S:W extracts media can subtract a considerable amount of silver in the form of precipitated AgCl, Ag_2CO_3, and Ag_2SO_4as salts or on the NPs surface, and that only a fraction of released ions will be available to interact with biological targets.

Finally, no significant amount of Ag was detected in PLLA-Ag samples both in ISO water and S:W extracts (below the limit of detection: LoD: 0.05 mg/L), suggesting that the low Ag content embedded into the fibres (Table S1), does not become available as free ions when in contact with investigated media.

3.3.3. *In the animals: Elemental quantification analysis*

For the exposure via soil, the animals exposed to Ag-Sigma showed a minor increase in Ag content after 28 days (Fig. 5A), while for AgHEC the Ag content increased with both time and concentration. The animals accumulated more Ag when exposed to AgHEC, particularly at 1000 mg Ag/kg soil. For PLLA-Ag there was little to no accumulation of Ag. Regarding the other quantified elements (Fig. S6), Fe was present in higher % in all of the Ag materials, without time and Ag dose dependency. On the other hand, Ni was virtually not detected. The % of Mg was relatively constant over time and across Ag materials, except for 1000 mg Ag/kg of Ag-Sigma after 21 days, and 1000 mg Ag/kg of AgHEC at 7 days, where it was very low. For Ag-Sigma exposed animals, the % of Cu and Zn increased with Ag dose up to day 14, followed by a sharp decrease after that (days 21 and 28). For AgHEC and PLLA-Ag

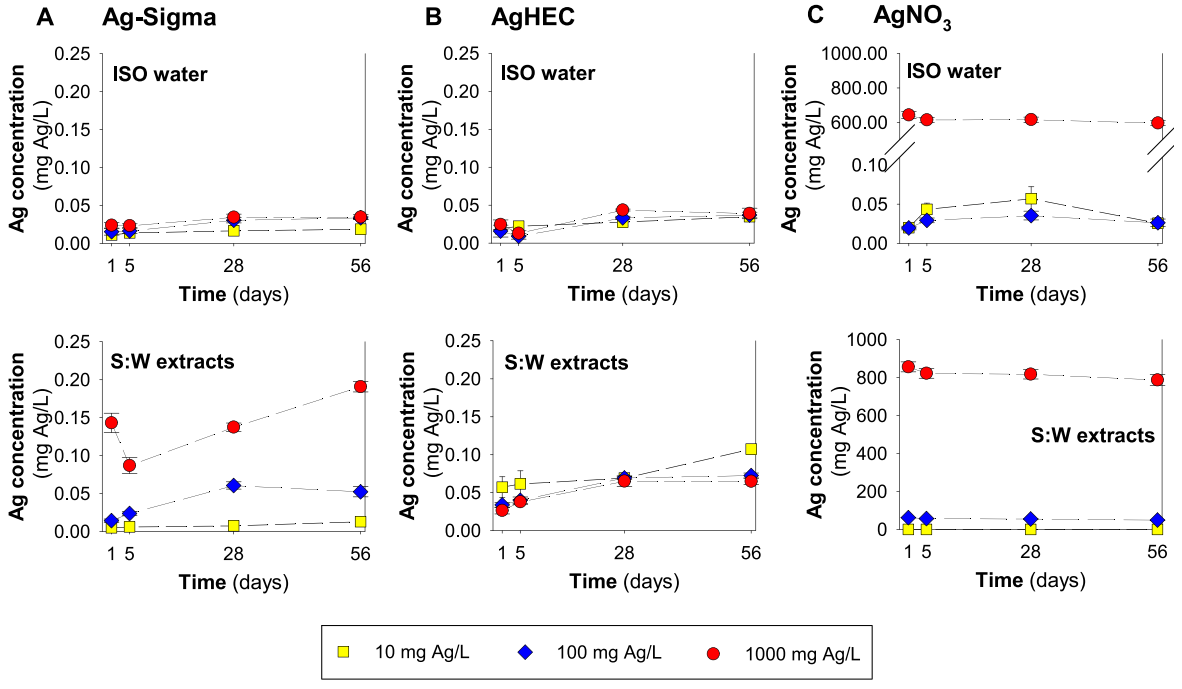

Fig. 4. Ag content released in the aqueous test media ISO water and S:W extracts after spiking with several concentrations of A) Ag nanopowder (Ag-Sigma and) B) hydroxyethyl cellulose coated Ag NPs (AgHEC), and C) $AgNO_3$, over time. Results are expressed as average ± standard error. The y axis was cut to improve visualization.

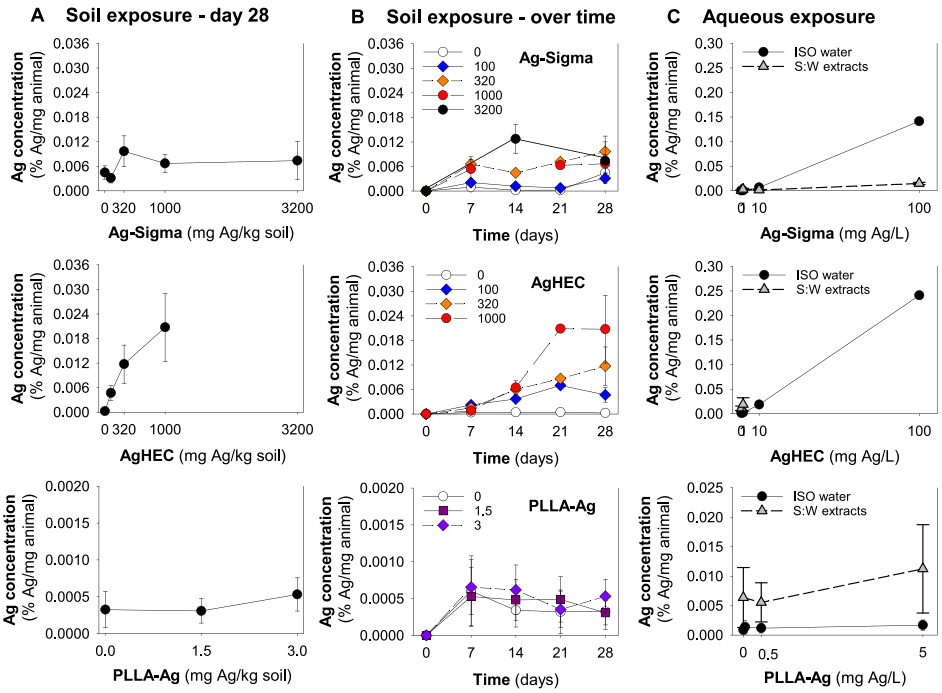

Fig. 5. Ag content in *Enchytraeus crypticus* exposed to Ag nanopowder (Ag-Sigma), hydroxyethyl cellulose coated Ag NPs (AgHEC), and Poly L-lactide nanofiber with embedded Ag NPs (PLLA-Ag) in A) LUFA 2.2. soil, after 28 days B) LUFA 2.2. soil, over 28 days and C) ISO water and S:W extracts, for 5 days. Results are expressed as percentage of Ag element (mg) over the total mass of animal (mg), as average ± standard error.

exposed animals, Cu was negligible.

For exposure via aqueous media, the same pattern of higher Ag accumulation for AgHEC was observed (Fig. 5C), although differentiated between ISO water and S:W extracts. For Ag-Sigma, the Ag content was higher for ISO water exposure compared to S:W extracts. For AgHEC it is not possible to discriminate due to animals' mortality at higher concentrations. For PLLA-Ag, the opposite pattern occurred, higher Ag accumulation occurred in S:W exposure compared to ISO water, although the data varied (high standard deviation). Similar to the animals exposed via soil, Ni was almost not detected in animals exposed via aqueous media (Fig. S7). The % of Zn are similar or even higher than Fe, and in S:W extracts, both Fe% and Zn% increased with Ag concentration, for all the Ag materials. Mg was not detected in animals exposed to PLLA-Ag via S:W extracts.

4. Discussion

Overall, and as commonly reported, $AgNO_3$ was confirmed the most toxic Ag form. Ag-Sigma and AgHEC were equally toxic to *E. crypticus* in soil, but exposure via S:W extracts revealed higher toxicity for AgHEC, related to a higher Ag accumulation. PLLA-Ag toxicity in aqueous media was higher than Ag-Sigma and slightly lower than AgHEC, despite the comparatively much lower accumulation of Ag by the animals exposed to PLLA-Ag. From the material characterisation aspect, Ag-Sigma forms large aggregates and unstable suspensions in aqueous media, while AgHEC forms stable suspensions of particles of nanometric size range. The Ag concentration in the aqueous media was low (<0.04 mg/L) and lower in ISO water when compared to S:W extracts. Interestingly, $AgNO_3$ formed AgCl precipitates in both aqueous media, these being in the nm size range in S:W extracts. The Ag concentrations measured in test media were overall higher in S:W extracts in comparison to ISO water, which could explain the higher toxicity observed.

The further discussion follows the structure as in the results, i.e., from soil tests to aqueous exposures.

The toxicity from exposure via soil for Ag-Sigma and AgHEC were similar (28 days AgHEC_EC50 = 686 mg Ag/kg soil; Ag-Sigma_EC50 = 446 mg Ag/kg soil), despite the primary size differences between materials (10–20 nm for AgHEC and 100 nm for Ag-Sigma), but there was a tendency to increase with prolonged exposure (56 days AgHEC_EC50 = 448 mg Ag/kg soil). These results were mostly in agreement with literature data for enchytraeids and earthworms exposed to other Ag NM powders despite size differences. The reproduction EC50 for *E. crypticus* exposed to 20–30 nm PVP (Polyvinylpyrrolidone)-coated and non-coated Ag NMs was 592 and 464 mg Ag/kg soil, respectively (Rodrigues et al., 2020), which was similar to the EC50 observed here for Ag-Sigma, both after 28 days (EC50 = 446 mg Ag/kg soil) and 56 days (EC50 = 500 mg Ag/kg soil). Similarly, the 56 days_EC50 for *Eisenia fetida* exposed to 50 nm Ag NMs was 445 mg Ag/kg soil (Novo et al., 2015). Also in agreement with our results, Shoults-Wilson et al. (2011a, 2011b) reported significant reduction in *E. fetida* reproduction at 1000 mg Ag/kg soil (10 and 30–50 nm Ag NMs), but not at 10 or 100 mg Ag/kg soil (although in a distinct design, e.g. concentration range and soil types). The results reported by Diez-Ortiz et al. (2015), for 50 nm Ag NM in LUFA 2.2. soil, indicated lower toxicity (EC50 = 1420 mg Ag/kg soil, although with overlapping 95 % confidence intervals (407–2432)) although the uncertainty in EC values is high. In terms of survival, *E. crypticus* seem to be more sensitive than *E. fetida*, for which no effects were reported up to 1000 mg Ag/kg soil (Shoults-Wilson et al., 2011a, 2011b) or up to 1758 mg Ag/kg soil (Novo et al., 2015). As in the above-mentioned publications, for size ranges from 10 to 50 nm there were similar effects to *E. fetida*. The fact that no toxicity was observed for PLLA-Ag exposure was not surprising given the comparatively much lower tested concentrations (up to 3 mg Ag/kg soil), far below the toxicity reported for Ag NM300K, the dispersed reference JRC material (Klein et al., 2011), which is among the most toxic Ag NMs [for *E. crypticus*, EC50 = 161 mg Ag/kg soil for Ag NM300K (Bicho et al., 2016a)].

Exposure through ISO water resulted in an overall low toxicity for all the Ag forms, both as acute (5 days in ISO water) and longer, after transfer to clean soil exposure. These results are in agreement with the previously reported for PVP-coated and non-coated Ag NMs (up to 100 mg Ag/L), the dispersed Ag NM300K (up to 40 mg Ag/L) and $AgNO_3$ (up

to 0.3 mg Ag/L), where no toxicity was observed using the same test design (Rodrigues et al., 2020). To note the non-monotonic response observed for AgHEC, which caused increased mortality at 10 mg Ag/L, but not at 100 mg Ag/L. The quantification of Ag in test media showed that the release within the first 5 days was similar for 10 mg Ag/L and 100 mg Ag/L, hence at a higher proportion at 10 mg Ag/L. This could be partially explaining the higher toxicity. Higher effects at lower concentrations have been reported before, e.g. for Ag NM300K (Bicho et al., 2016a) and for Ni NM via soil exposure (Santos et al., 2017). One possible explanation was the lower agglomeration with consequent higher dissolution at the lower concentrations. The inverse relation between particles' size and dissolution is known, i.e., smaller particles tend to dissolve more (Sotiriou and Pratsinis, 2010; Zhang et al., 2011). Nevertheless, the surviving animals recovered and were able to reproduce after 21 days in clean soil, suggesting that the stress induced by AgHEC could be managed by a fraction of the population and possibly reversed via defence mechanisms. This was also the case for *E. crypticus* exposed to $AgNO_3$, for which an increased mortality at 5 mg Ag/L (ISO water) was recovered after transfer to clean soil. Comparatively to Ag-Sigma results, a study from Topuz and van Gestel (2015) reported higher toxicity for Ag NMs, using inert quartz sand and reconstituted water as test media (5 days, 25 mg Ag/L < $LC50_{(citrate\ (Cit)\text{-}coated\ Ag\ NM)}$ < 50 mg Ag/L; 6.25 mg Ag/L < $LC50_{(PVP\text{-}coated\ Ag\ NM)}$ < 12.5 mg Ag/L). To note that Topuz and van Gestel (2015) test media did not include chloride, which is part of the ISO reconstituted water used, because it can influence Ag speciation. This might have contributed to the differences in toxicity reported. Further, interaction of Ag NMs with the sand matrix cannot be excluded either.

Overall, exposure via S:W extracts caused higher toxicity compared to ISO water. This higher toxicity in exposure via S:W extracts was reported previously for europium polyoxometalate encapsulated in silica nanoparticles (Eu-POM/SiO_2 NPs), using the same test design (Bicho et al., 2016b). The opposite, i.e., higher toxicity in ISO water, was reported for TiO_2 NMs (Gomes et al., 2015a) hence the interactions are NM specific, i.e., depend on the involved materials. In the case of Ag-Sigma and AgHEC, there was higher dissolution (higher Ag measured) in the S:W extracts compared to ISO water, hence this must be the source and reason for the higher toxicity. The higher dissolution is probably due to the presence of organic molecules complexing the NMs which may facilitate the oxidation of Ag by shifting the equilibrium (Loza et al., 2014).

Interestingly, the higher Ag concentration in the media was not always associated with higher Ag content in the animals (which was higher for the animals exposed via ISO water). This indicates that while the Ag content in the animals reflects Ag NMs uptake, the higher Ag concentration in the media caused higher toxicity, hence it seems to be an availability issue, and the available fraction of Ag ions could promote more than one mechanism.

Ag-Sigma was the least toxic in S:W extracts (5 days LC50 = 299 mg Ag/L S:W extracts), but the effects persisted and tended to increase after 21 days of post-exposure in clean soil (21 days LC50 = 166 mg Ag/L, and EC50 = 108 mg Ag/L), which could be due to a bioaccumulation effect. PLLA-Ag, which caused no effects via ISO water exposure, was ca. 60 times more toxic than Ag-Sigma (5 days LC50 = 4.9 mg Ag/L S:W extracts for PLLA-Ag). The higher toxicity of PLLA-Ag cannot be explained by Ag accumulation (lower for PLLA-Ag exposed animals), but its aspect-ratio might have a key role. PLLA-Ag consists of Ag NMs (Ag-Sigma) embedded into PLLA microfibers, a biodegradable polymer containing fibres with a mean diameter of ~4 μm and a thickness of the electrospun layer of 50 μm. One of the major concerns about fibrous (nano) materials (e.g. nanofibers, nanowires, nanotubes) arises from its well-known potential to cause pulmonary inflammation, fibrosis, and cancer, similar to asbestos mineral fibres. Most of the toxicity data focus on the immune response and/or respiratory exposure mimicking cell lines, revealing for instance that cellulose nanofibers cause inflammation although less severe than carbon nanotubes (Ilves et al., 2018) or asbestos (Park et al., 2018). For TiO_2, nanofibers were more toxic (in vitro) than TiO_2 nanoparticles (Allegri et al., 2016), and longer TiO_2 fibres were more toxic than shorter (as the combined result of a more severe damage of epithelial cells and a less efficient clearance attributable to ineffective phagocytosis). Hence, the production of shorter fibres, compared to longer, could constitute a SbD alternative to reduce or mitigate TiO_2 nanofibres risks (Bianchi et al., 2020). In soil, copper (Cu) nanowires were more toxic to *E. crypticus* than Cu NPs (EC50 of 1613 and 2748 mg Cu/kg soil for Cu nanowires and NPs, respectively) (Gomes et al., 2018a). We cannot conclude about the toxicity of PLLA-Ag versus Ag-Sigma in soils at comparable concentrations in the current study. However, results from S:W extracts test showed a much higher toxicity for the fibrous PLLA-Ag. If the toxicity is (partially) induced by the (PLLA) fibres alone it is unknown, but the presence of organic matter (as in S:W extracts but not in ISO water) seems to have an important role in the observed toxicity, probably by facilitating the fibres uptake (animals exposed to PLLA-Ag in S:W extracts accumulated more Ag). The toxicity of PLLA-Ag induced via the S:W extracts short-term exposure persisted after transfer to clean soil during a longer exposure period (5 days LC50 = 4.9 mg Ag/L S:W extracts; 21 days LC50 and EC50 = 5.8 and 5.4 mg Ag/L). This could be related to persistent/long-term inflammation as caused by the low clearance (thus persistence) of the fibres inside the animals. That seems to be also the case for the PLLA-Ag via ISO water exposure, which although not inducing acute toxicity (maybe due to the lower uptake), it caused a chronic toxicity, i.e. a decrease in reproduction after 21 days in clean soil (21 days EC20 and EC50 = 3.2 and 11.2 mg Ag/L, based on pre-exposure concentrations).

AgHEC was the most toxic NM via S:W exposure (5 days LC50 = 1.9 mg Ag/L S:W extracts). Although the dry AgHEC had an irregular, fluffy-spongy aspect (as also observed when spiking the soil, Fig. S1 and S5), it forms stable aqueous dispersions of spherical Ag NPs homogeneously coated with cellulose (not with a fibrous shape), thus the differences in toxicity compared to Ag-Sigma are not likely due to aspect-ratio differences. HEC, and other cellulose ethers, are frequently used as polymeric matrices in controlled release formulations, thus the release of Ag from AgHEC would take longer to occur than for Ag-Sigma (pristine NPs). This was supported by our in media characterisation results, where AgHEC showed less Ag release than Ag-Sigma, particularly in S:W extracts. Further, the dissolution of AgHEC was similar across concentrations, indicating that the cellulose-based coating (HEC) must act as a protective layer, mitigating the complexation action of the organic ligands dispersed in the medium. This is also in agreement with a study comparing pristine Ag NPs with Ag NPs with different coatings: PVP, citrate and HEC, showing the lower Ag^+ release for AgHEC (Marassi et al., 2018). Marassi et al. (2018) also showed that AgHEC was the best candidate to use in medical devices because it presented long-lasting antibacterial/antiseptic activity with lower toxicity and better recovery using a human skin model (in vitro). Our results showed however higher acute toxicity (100 % mortality for 100 mg Ag/L after 24 h exposure, and for 10 mg Ag/L after 48 h) [higher toxicity was only detected for $AgNO_3$ (100 % mortality above 0.5 mg Ag/L after 24 h, and for 0.05 mg Ag/L after 48 h)]. The pH of AgHEC suspensions in S:W extracts was high, and increased with concentration (Table S5), although this was not likely the cause of toxicity since a similar pH occurred in ISO water media (Table S4), and without significant effects to the animals. Assuming that this is not solely related to Ag^+ release in the test media, which was higher for Ag-Sigma, the higher uptake due to the HEC coating is a possibility, as also promoted by its interaction with the organic matter present in S:W extracts (note that little to no effects were reported in ISO water). In the case of AgHEC we could not determine Ag accumulation in animals exposed above 1 mg Ag/L in S:W extracts due to 100 % mortality but, at 1 mg Ag/L the Ag content was higher for animals exposed through S:W extracts compared to ISO water. If Ag accumulation was even higher at 10 and 100 mg Ag/L, increasing with concentration, that would explain the high toxicity observed. Once inside the cells, Ag^+ might have been released from NM in higher

amounts (trojan horse effect) inducing higher toxicity. The surviving organisms (exposed to 1 mg Ag/L) did not show effects after transfer to clean soil (i.e., their survival or reproduction was not affected), which is in line with the results from Marassi et al. (2018) in human cell lines. $AgNO_3$ was the most toxic Ag form, via soil or aqueous exposure. This follows previous literature data for *E. crypticus* (Rodrigues et al., 2020). For the aqueous exposure, $AgNO_3$ was more toxic to *E. crypticus* via S:W extracts than via ISO water (LC50 = 13.8 mg Ag/L ISO water, and LC50 < 0.05 mg Ag/L S:W extracts). The formation of AgCl precipitates was observed in both aqueous media and detected by DLS, although in the micrometre size range in ISO water and in the nanometric size range in S:W extracts (possibly due to Ostwald ripening process of AgCl aggregates (Vollmer et al., 2014)). Hence, the formation of nano-AgCl complexes in S:W extracts could explain the higher toxicity in this media. Further, in S:W extracts, the Ag release to the media was higher than in ISO water, which could also be related to the formation of smaller AgCl aggregates. Overall, results indicate that the sustainable AgHEC (in terms of synthesis) is the most bio-active composite. This result opens the way to its use as antimicrobial ingredients, aiming for a concentration range where the material is toxic for microorganisms but not for non-target organisms.

5. Conclusions

The toxicity of Ag-Sigma and the SSbD alternative AgHEC to *E. crypticus* was similar, via LUFA 2.2 soil exposure. However, exposure through the simplified surrogate soil:water extracts media revealed that AgHEC was 150 times more toxic than Ag-Sigma and 2.5 times more toxic than the PLLA-Ag fibres, probably related to higher uptake of NMs. Materials characterisation in the media showed that the higher toxicity was associated to smaller aggregate size and higher stability of the suspensions. This resulted in higher % of Ag concentration, particularly in S:W extracts, for which higher toxicity was observed. Interestingly, $AgNO_3$ forms AgCl precipitates (μm to nm size ranges in ISO water and S:W extracts respectively), suggesting that also for $AgNO_3$, animals can be exposed to nano Ag. Overall, AgHEC was not a SSbD alternative to Ag-Sigma or PLLA-Ag in the environment, considering this non-target soil living species. There was a good correlation between measurement of materials concentration in media and toxicity. Further, the ability to test *E. crypticus* in a range of media with increasing complexity (soil, soil:water extracts, water) provided an excellent source to interpret results. Hence, we recommend this testing approach for a comprehensive interpretation and results of ecotoxicity of nano or advanced materials.

CRediT authorship contribution statement

Susana I.L. Gomes: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Ilaria Zanoni:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Magda Blosi:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Anna L. Costa:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization. **Danail Hristozov:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Janeck J. Scott-Fordsmand:** Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Mónica J.B. Amorim:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

This study was supported by the European Commission Projects BIORIMA (H2020-NMBP-2017, GA No. 760928). Further support within NANOINFORMATIX (H2020-NMBP-14-2018, GA No. 814426), NANORIGO (H2020-NMBP-13-2018, GA No. 814530) and CESAM [UIDB/50017/2020 + UIDP/50017/2020 + LA/P/0094/2020], via FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. S. Gomes is funded by FCT, I.P. via a research contract under the Scientific Employment Stimulus - Individual Call (CEEC Individual) - 2021.02867.CEECIND/CP1659/CT0004.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171860.

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