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Responses to silver nanoparticles and silver nitrate in a battery of biomarkers measured in coelomocytes and in target tissues of *Eisenia fetida* earthworms

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ABSTRACT

The current use and development of applications with silver nanoparticles (Ag NPs) could lead to potential inputs of these NPs to soils. Consequently, it is crucial to understand the ecotoxicological risks posed by Ag NPs in the terrestrial compartment. In the present investigation, the effects produced by PVP-PEI coated Ag NPs were assessed in *Eisenia fetida* earthworms in comparison with the soluble form ($AgNO_3$). Earthworms were exposed for 1, 3 and 14 days to a range of sublethal concentrations of Ag (0, 0.05 and 50 mg/kg) and at each exposure time, apart from mortality and weight loss of individuals, metallothionein (MT) protein concentration and catalase (CAT) activity were quantified in earthworm tissues. In addition, cellular and molecular level endpoints (cell viability, absolute and relative trophic indices and transcription levels of catalase-*cat*- and metallothionein-*mt*-) were measured in coelomocytes extruded from exposed earthworms. Despite the lack of effects in traditional endpoints (mortality and weight loss), Ag NPs and $AgNO_3$ posed changes at lower levels of biological complexity (biochemical, cellular and molecular levels). Both Ag forms induced similar changes in the metal detoxification mechanism (MT, *mt*) and in the antioxidant response system (CAT, *cat*) of *E. fetida*. In contrast, Ag form dependant cytotoxicity and subpopulation ratio alterations (eleocytes/amoebocytes) were recorded in extruded coelomocytes. Complementarily, the use of coelomocytes to assess molecular level endpoints represented a relevant alternative for development of non-invasive biomarkers.

1. Introduction

The wide range of current and potential future applications exhibited by silver nanoparticles (Ag NPs) has made them one of the most commonly used nanomaterials (Dubey et al., 2015; Vance et al., 2015). Due to these applications and to the massive disposal of sewage sludge released from Waste Water Treatment Plants (WWTP, one of the major sources of Ag NPs in biosolids), Ag NPs might have the potential to severely affect soil health (Shoults-Wilson et al., 2011; Tourinho et al., 2012). However, the potential risk of Ag NPs in soils has been poorly investigated in comparison with aquatic environments. Even if fewer studies have involved the effects of Ag NPs on terrestrial organisms, the number of studies carried out with earthworms has increased during the last five years (Diez-Ortiz et al., 2015a, 2015b; García-Velasco et al., 2016; Gomes et al., 2013, 2015; Hayashi et al., 2012; Kwak and An, 2015; Shoults-Wilson et al., 2011; Tsyusko et al., 2012).

Earthworms play an important role in terrestrial ecosystems (*e.g.* decomposition and nutrient recycling) and therefore, the study of effects exerted by Ag NPs on them is crucial to understand the potential impacts of NPs in soils. In this context, standard toxicity tests (OECD, 1984, 2004) with *Eisenia fetida* earthworm are aimed to address traditional endpoints such as survival or weight loss in order to calculate different toxicity indices (LC_x and EC_x). Furthermore, tissue, cellular or molecular level biomarkers could be also quantified in target tissues of *E. fetida* in order to assess the exposure degree or the toxic effects of pollutants. For instance, metallothioneins (MTs), low molecular weight proteins, with high cysteine content (up to 30%) that enables to bind a variety of metal atoms (Asensio et al., 2007; Brulle et al., 2006), participate in homeostasis of essential metals and in the

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detoxification of toxic trace metals (Brulle et al., 2006) and may prevent oxidative stress (Ribeiro et al., 2015). Ag NPs are known to cause oxidative stress in terrestrial invertebrates by the production of highly reactive oxygen species (ROS) that can damage cell components including DNA, proteins and membranes (Yang et al., 2011). Cells, in order to protect themselves from ROS, have developed complex defense systems including non-enzymatic scavengers and antioxidant enzymes such as catalase (CAT). A change in CAT activity is an indicator of a cellular lesion after exposure to chemicals, and thus it is considered as an early environmental stress biomarker (Asensio et al., 2013; Gomes et al., 2015).

Biomarkers can be measured in earthworm tissues or even in target cells as coelomocytes. Coelomocytes are the immune cells of earthworms and play a pivotal role in recognition and elimination of foreign materials and are involved in clotting and wound healing (Cooper, 2002; Kurek et al., 2007). Coelomocytes compose a heterogeneous cellular group that circulates in fluid-suspension in the coelomic cavity. Based on cytomorphometric, ultrastructural and cytochemical properties three cell types are distinguished: eleocytes (derived from the chloragogenous cells that surround the digestive epithelium), hyaline amoebocytes and granular amoebocytes or granulocytes (for detailed descriptions see Adamowicz (2005)). Changes in coelomocytes viability and subpopulation ratios in earthworms exposed to xenobiotics or subjected to different types of stress reflect alterations in the earthworms immune response and in the general health status (Di Marzio et al., 2005; Homa et al., 2003; Irizar et al., 2015b). Hence, these cellular parameters have been proposed as biomarkers of general stress in soil toxicity assessment (Homa et al., 2003; Irizar et al., 2015b; Olchawa et al., 2006). Regarding lower levels of biological organization, Ag NPs are known to alter the transcription of genes involved in the abovementioned pathways in *E. fetida*: oxidative stress, detoxification and immune signaling (Hayashi et al., 2013; Tsyusko et al., 2012). Transcription levels of target genes such those encoding CAT or MT have been easily measured in earthworm tissues (Asensio et al., 2007; Brulle et al., 2006; Irizar et al., 2014b). However, the utilization of immune cells (coelomocytes) to assess molecular level endpoints would represent a relevant alternative for the development of non-invasive biomarkers in more controllable and reproducible test systems than whole animals.

The aim of the present investigation was to assess the toxicity of PVP-PEI coated Ag NPs in earthworms, *E. fetida*, in comparison with the soluble form of the metal ($AgNO_3$). For this purpose, earthworms were exposed for 1, 3 and 14 days to a range of sublethal concentrations of Ag (0, 0.05 and 50 mg/kg) in the form of Ag NPs and $AgNO_3$. At each exposure time, apart from mortality and weight loss of individuals, MT protein concentration and CAT activity were quantified in earthworm tissues. In addition, cellular and molecular level endpoints (cell viability, absolute and relative trophic indices and transcription levels of *cat* and *mt* genes) were measured in coelomocytes extruded from exposed earthworms.

2. Materials and methods

2.1. Test species

Eisenia fetida earthworms (350–500 mg fresh weight) used for the experiments were healthy adults, clitellated and obtained from the stock population provided by a commercial dealer (LOMBRICOR S.C.A., Córdoba, Spain). Earthworms were maintained in the laboratory under controlled conditions of temperature (19 ± 2 °C), darkness and constant humidity. As food source medication-free horse manure was provided when required.

2.2. Test substances

Polyvinylpyrrolidone-polyethylenimine (PVP-PEI, 3.35:1) coated silver nanoparticles (NP Ag-2106W) were purchased from NANOGAP (SUB-NM-POWDER, S.A., A Coruña, Spain). Ag NPs were water dispersed (10 g Ag/L with 104 g PVP-PEI/L), 5.08 ± 2 nm average size and with a Z-potential of 18.6 ± 7.9 mV. Particle size distribution and zeta potential determinations through Dynamic Light Scattering were provided by NANOGAP CoA. High grade (> 99% purity) $AgNO_3$ was purchased from Sigma-Aldrich.

2.3. Artificial soil preparation, contamination and characterization

The OECD artificial soil was prepared following the OECD guideline 207 (OECD, 1984). The artificial soil contained 70% sand (50% of particles were between 50 and 200 μm), 20% kaolin clay and 10% sphagnum peat sieved at 2 mm. pH was adjusted to 6.0 ± 0.5 by addition of 0.01% calcium carbonate. Dry constituents were mixed, placed in glass containers and moistened to 40% of their water holding capacity (WHC, 21.91%) with suspensions of Ag NPs and solutions of $AgNO_3$ in distilled water or with distilled water in the case of the control group. Two sublethal concentrations (0.05 and 50 mg Ag/kg soil) were chosen according to previous experiments (García-Velasco et al., 2016). After spiking with the corresponding silver form, experimental soils were thoroughly mixed to ensure a homogeneous distribution of the metal. Then soils were stabilized during 3 days before adding earthworms previously acclimated (24 h) to OECD soil. Earthworms (n = 20) were exposed to unpolluted soil (control) and to soils spiked with Ag NPs or $AgNO_3$ during 1, 3 and 14 days. At the end of each Ag exposure, weight loss was assessed in earthworms and Ag quantification and pH measurements were carried out in experimental soils. The real concentration of Ag in soils was quantified following the EPA 3051A method and analyzed in Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies) in the Central Analysis Service (SGIker) of the University of the Basque Country following USEPA directions (USEPA, 2007). Detection limit (DL) was 0.03 mg/kg. For the measurements of the pH an adaptation of the ISO 10390: 2005 “Soil Quality – Determination of pH in water” was followed.

2.4. Concentration of MTs

MTs concentration was determined in earthworms by the spectrophotometric method described by Viarengo et al. (1997). In order to perform pools, the post-clitellar portion of 3 earthworms were weighed and homogenized in three volumes of 0.5 M sucrose and 20 mM Tris–HCl buffer (pH 8.6) containing 0.006 mM leupeptine and 0.5 mM phenylmethylsulfonilfluoride, as an antiproteolytic agents, and 0.01% β-mercaptoethanol, as a reducing agent. Homogenates were ultracentrifuged (30.000 × *g*, 20 min, 4 °C) and precipitated with ethanol/chloroform. Three pools were done per treatment and exposure time. MTs concentration was quantified by spectrophotometric titration of the sulfhydryl residues using the Ellman's reagent (5,50-dithiobis-2-nitrobenzoic acid) with reduced glutathione (GSH) as standard. Samples were centrifuged for 5 min (530 × *g*, 4 °C) and the supernatant (300 μL) was added in 96-well microplate wells. Each sample was replicated four times. Finally, absorbance was measured at 412 nm in a microplate reader Multiskan Thermo Scientific Spectrophotometer. Data were expressed as μg MTs/g earthworm wet weight (ww).

2.5. CAT activity

CAT (EC 1.11.1.6) activity was determined measuring decrease of absorbance at 240 nm due to hydrogen peroxide consumption (Claiborne, 1985). The pre-clitellar portion of 5 earthworms were weighed and homogenized in five volumes of homogenization buffer (TVBE pH 7.4) in order to obtain pools. Two pools per treatment and exposure time were used. Absorbance was measured in 96-well UV Flat Bottom microplates and using a microplate reader Multiskan Thermo

Scientific Spectrophotometer. Four replicates were added per sample and a standard curve was also included in the plates. Total protein content was estimated according to Lowry et al. (1951) using bovine gamma globulin as standard. CAT activity was expressed as mM of H_2O_2/mg of protein/min.

2.6. Coelomocyte extrusion and cell viability

Before the extrusion earthworms were rinsed in tap water, weighted and placed on a damp paper towel overnight to allow them to void gut contents. Coelomocytes were collected using a non-invasive extrusion method (Di Marzio et al., 2005). Briefly, four pools (of 5 organisms) per treatment and exposure time were placed into centrifuge tubes containing 2 mL of 5% ethanol saline solution/individual and incubated for 1 min. Coelomic fluid containing extruded cells was diluted with calcium and magnesium free phosphate buffered saline (PBS), washed twice, and centrifuged at 530 × *g* at 4 °C during 10 min. Final pellets were resuspended in 2 mL of PBS and cells were counted using a counting chamber (Neubauer hemocytometer). Cytotoxicity was expressed as the percentage of non-viable cells measured with 0.4% Trypan Blue. Additionally, one hundred cells were counted on each slide and three replicate slides were analyzed per pool for characterization of the population of coelomocytes. Extruded coelomocytes were characterized according to their morphology as eleocytes or amoebocytes according to Adamowicz (2005). Absolute Trophic Index Earthworm (ATIE) and Relative Trophic Index Earthworm (RTIE) were determined as follows: ATIE, En/Cn, where *En* is the average number of total eleocytes per individual / mL of coelomic fluid and *Cn* is the average number of total coelomocytes per individual / mL of coelomic fluid and RTIE, ATIE/wwf, where *wwf* is wet weight without feces.

2.7. cat and mt transcription levels in extruded coelomocytes

The same coelomocyte pools used to record cell number and viability (4 pools- from 5 individuals- per treatment and exposure time) were employed to quantified *cat* and *mt* transcription levels. Coelomocytes (2×10^6 cell pellets) were homogenized in TRIzol® (Invitrogen, Thermofisher Scientific USA) using silica beds in a HYBAID RiboLyser (FP120-HY-230) for 45 s at maximum speed. Total RNA was extracted following the manufacturers protocol (TRIzol® method). RNA purity and integrity were spectrophotometrically checked. RNA was purified using the RNeasy kit (Qiagen) following manufacturers indications and 1 μg of total RNA was retro-transcribed into cDNA using the AffinityScript Multiple Temperature kit (Agilent Technologies). *Cat* and *mt* transcription levels were quantified in a 7300 Real Time PCR System (Applied Biosystems, Thermofiher Scientific) using FastStar Universal SYBR Green Master mix (Roche). Each reaction (final volume 20 μL) contained 2 μL of template (previously diluted at 1/100), 0.25 μL of 25 pmol primer pair (Table 1), 7.75 μL of RNAse free water, and 10 μL of SYBR Green 2x. qPCRs were run as follows: 2 min at 50 °C, 10 min at 95 °C; 40 cycles at 95 °C (15 s) followed by each annealing temperature (Table 1) (45 s). Efficiency was determined running a standard curve and specificity of each reaction was determined by the melting curve where a single peak was identified in all dissociation curves, confirming the production of a single amplicon per primer set. In all cases, a control without template was run for quality assessment. The specific amplification of each amplicon was also checked by sequencing both PCR products. Relative Quantification (RQ) of the transcription levels was calculated using a plate calibrator to obtain the ΔCT, the efficiency (E) of the PCR and the amount of cDNA (in ng) used in each reaction:

$$RQ = (1 + E)^{-\Delta CT}/\text{ng } c\text{DNA}$$

Amount of cDNA was determinate by using QuantiT OliGreen ssDNA assay Kit following manufacturer's procedure. RQ values were represented relative to the average of control earthworms in each exposure time (1, 3 and 14 days).

2.8. Statistical analysis

Normal distribution of data was assessed using the Shapiro-Wilk's test and homogeneity of variance was tested using the Bartlett's test. Significant differences ($p < 0.05$) with respect to the control were based on the non-parametric Kruskal-Wallis test followed by the Dunn´s post hoc test. Differences between Ag forms were explored with Student´s t (parametric) and Mann-Whitney U (non-parametric) tests, being in this case significant differences established at $p < 0.001$. All statistical analysis was performed using Statistica v. 8 (StatSoft).

3. Results

3.1. Ag concentration and pH of soils

Real concentrations of Ag in experimental soils were similar to nominal concentrations with the exception of the 0.05 mg $AgNO_3$/kg experimental group that showed 0.53 mg Ag/kg as real concentration (Table 2). Soil pH remained around 6 during the experiment for all the exposure groups.

3.2. Weight loss

Control and exposed earthworms lost similar weight (15–17%) during the experiment, regardless of the Ag form (Ag NPs and $AgNO_3$) and time (1, 3 and 14 days).

3.3. Concentration of MTs

MTs concentration did not change during the experiment time in control earthworms. MT levels significantly increased with respect to controls after exposure to both concentrations (0.05 and 50 mg/kg) of Ag NPs and $AgNO_3$ ($p < 0.05$; Fig. 1). The major increases were recorded at day 3. No differences in MT concentration were found between Ag forms at day 1. After 3 days of exposure, MT concentrations were significantly higher in $AgNO_3$ treatments in comparison to Ag NPs ones. At day 14 MT concentrations appeared to be significantly higher after exposure to Ag NPs at the highest dose (50 mg/kg).

3.4. CAT activity

CAT activity did not show alterations during the experiment in

Table 1
Primer sequences (F: forward and R: reverse), annealing temperature (°C) for each primer pair and expected length of each PCR product for the specific amplification of metallothionein (*mt*) and catalase (*cat*) in earthworms.

Gen	Gen Bank access	Primer sequence	Annealing T (°C)	Amplicon size (bp)
mt	AJ236886	F: 5´-AAATGCTCGGCTGGTTCGT-3´ R:5´- TGATGACAGAGTTCCGTATTTC-3´	55.5	103
cat	DQ286713	F:5´- GCCGACGGAGAAGCTGTGTA-3´ R: 5´-TAAAGGTCACGGGTCGCATAG-3´	59	125

Table 2
Nominal and real Ag exposure concentrations (mg /kg soil) and pH of experimental soils. Detection limit (DL) was 0.03 mg/kg.

Nominal concentration (mg/kg)		Real concentration (mg Ag/kg)	pH
Control	0	0.05	5.93
Ag NPs	0.05	0.09	5.99
	50	48.40	5.89
$AgNO_3$	0.05	0.53	6.00
	50	50.30	6.11

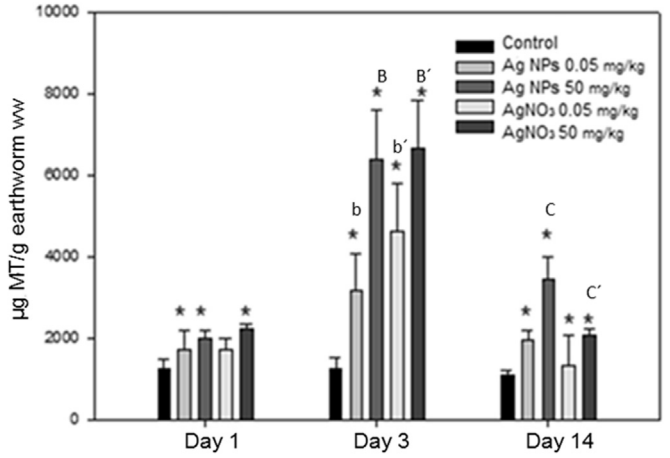

Fig. 1. Concentration of metallothioneins (MTs) in organisms exposed to Ag NPs and AgNO3 (0.05 and 50 mg/kg) and unpolluted soils (control) for 1, 3 and 14 days. Mean values and standard deviations are shown. *Statistically significant differences with respect to the control group ($p < 0.05$). Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.001$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

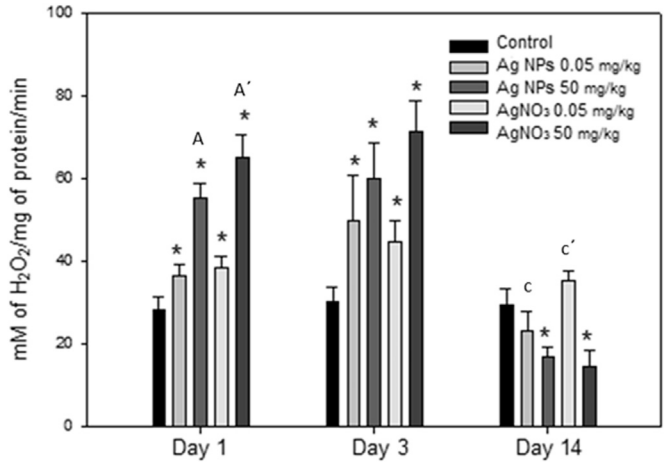

Fig. 2. Catalase activity (CAT) in organisms exposed to AgNPs and $AgNO_3$ (0.05 and 50 mg/kg) and unpolluted soils (control) for 1, 3 and 14 days. Mean values and standard deviations are shown. *Statistically significant differences with respect to the control group ($p < 0.05$). Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.001$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

control organisms (Fig. 2). In earthworms exposed to both concentrations (0.05 and 50 mg/kg) of Ag NPs and $AgNO_3$ CAT activity was significantly enhanced in comparison to controls up to day 3 (Fig. 2). After 14 days of exposure, activity was reduced, especially after exposure to the highest dose of both Ag forms (Fig. 2). CAT activity was significantly higher in earthworms exposed to $AgNO_3$ than to Ag NPs at day 1 (at 50 mg/kg concentration) and day 14 (0.05 mg/kg concentration).

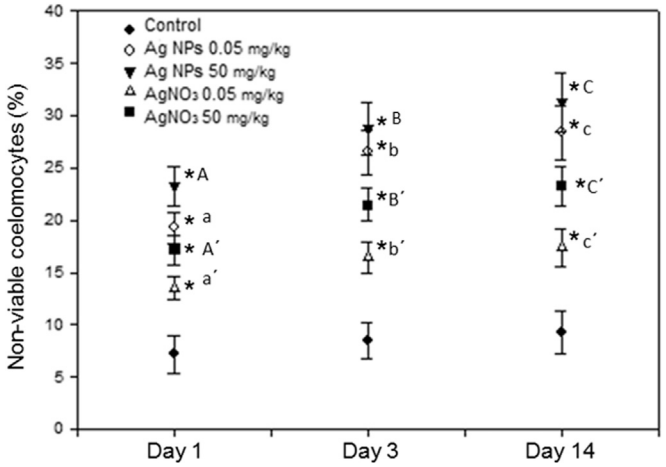

Fig. 3. Cytotoxicity (Trypan Blue 0.4%) after 1, 3 and 14 days of *in vivo* exposure to Ag NPs and $AgNO_3$ (0.05 and 50 mg/Kg) and unpolluted soils (control). *Statistically significant differences respect to the control group ($p < 0.05$). Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.001$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

3.5. Cytotoxicity

Percentage of non-viable coelomocytes in controls was stable along the experimental period. Percentage of non-viable coelomocytes increased significantly in earthworms exposed to Ag (NPs or salts) (Fig. 3). Exposure to Ag NPs produced significantly higher cell mortality than $AgNO_3$ for the same exposure concentration and times.

3.6. Trophic indexes, ATIE and RTIE

The number of eleocytes (Fig. 4) in control earthworms and in earthworms exposed to the lowest concentration (0.05 mg/Kg) of Ag NPs and $AgNO_3$ was similar and remained stable all along the experiment (Fig. 4). However, after 3 and 14 days of exposure to the highest concentration of Ag NPs (50 mg/kg) the total number of eleocytes increased significantly in comparison to controls. Exposure to $AgNO_3$ significantly decreased the number of eleocytes in comparison to control groups after 1 and 3 days of exposure to the highest dose (50 mg/kg). When comparing Ag forms, earthworms exposed to $AgNO_3$ showed significantly lower ATIE and RTIE levels.

3.7. cat and mt transcription levels in extruded coelomocytes

Overall, *cat* and *mt* transcription levels were higher in coelomocytes extruded from earthworms exposed to both forms of Ag (Ag NPs and $AgNO_3$) for 1 and 3 days in comparison to control coelomocytes (Fig. 5). These differences were enhanced after the exposure to the highest concentration of Ag (50 mg/kg). After 14 days, Ag NPs caused increase of *cat* transcription levels while no significant differences were found in *mt* transcription levels. After exposure to $AgNO_3$, both genes were significantly up-regulated at low doses (0.05 mg/Kg) while at the highest exposure concentration a significant inhibition was observed for *mt* (Fig. 5A). In the case of *cat*, transcription levels decreased up to the control values after 14 day exposure to the highest dose of $AgNO_3$ (Fig. 5B). Alterations in *mt* and *cat* transcription levels were significantly higher after $AgNO_3$ exposure than after Ag NPs exposure, mainly at days 1 and 14 and after exposure to the low dose.

4. Discussion

An adequate spiking and homogenizing procedure is one of the most crucial issues when characterizing the toxicity of pollutants in soils (Waalewijn-Kool et al., 2014). Presently, Ag concentrations measured

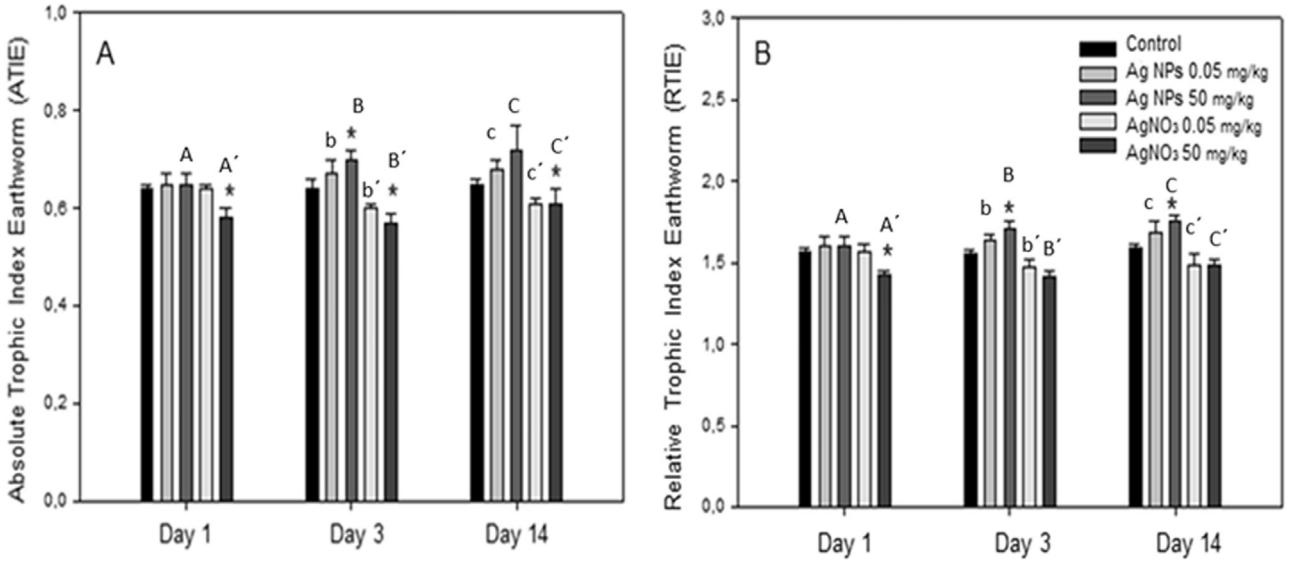

Fig. 4. A. Absolute Trophic Index Earthworm (ATIE) and 4.B. Relative Trophic Index Earthworm (RTIE) after 1, 3 and 14 days of exposure to Ag NPs and $AgNO_3$ (0.05 and 50 mg/Kg) and unpolluted soils (control). Values are represented as mean values of six cell counts and standard deviations. *Statistically significant differences with respect to the control group ($p < 0.05$). Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.001$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

in both Ag NPs and $AgNO_3$ spiked soils did not differ from the nominal concentrations, with the exception in 0.05 mg $AgNO_3$/kg treatment (0.53 mg Ag/kg as real concentration, probably due to inhomogeneity/contamination of the soil sample collected), indicating overall validity of the spiking protocol used herein. In this context, it is noteworthy that chemical analyses carried out on experimental soils ensured the exposure of earthworms to different known concentrations of Ag (NPs or salt) for up to 14 days. This exposure may exert physiological responses at different levels of biological complexity, possibly altering earthworm fitness, and ultimately changing their populations or community densities. Aiming to measure these effects, standard toxicity tests (OECD, ISO) with *E. fetida* earthworms are based on short and long-term experiments and traditional endpoints (Moser and Römbke, 2009). However, presently earthworms appeared to be unaffected at high levels of biological organization (organism level) since severe weight losses (> 20%) were not recorded after Ag NPs and $AgNO_3$ exposures. It seemed that exposure concentrations and duration (or both) were not high enough to produce significant somatic effects at the organism level. Accordingly, García-Velasco et al. (2016) in a previous works dealing with Ag NPs toxicity in earthworms proved that the EC_{50} for weight loss after 14 days of exposure was higher than the highest dose used in the present work ($EC_{50} = 57.62$ mg Ag NPs/kg).

Complementary to the classical toxicity endpoints, changes in health status can be detected at lower levels of biological complexity, which can forecast effects in more ecologically relevant parameters. In fact, even if the weight loss of earthworms remained unaltered after exposure to both silver forms, MT concentrations significantly increased in comparison with controls after exposure to low and high doses. This might suggest a possible activation of the metal detoxification mechanism that involves these metal quenching proteins, as Ribeiro et al. (2015) found for *Enchytraeus crypticus* after exposure to both silver forms. Hence, Ag would be selectively bound to MTs and the resulting Ag-MT complexes would be sequestered into lysosomes (García-Velasco et al., 2016; Marigómez et al., 2002). Thus, MT could participate in Ag removal, helping to prevent oxidative stress, mainly at short exposure times (day 3). In fact, biochemical responses are known to be time dependant, and therefore, 3 days could be enough to scavenge Ag^+. A similar pattern was reported for *E. fetida* after exposure to carbon nanotubes, where MT concentration increased significantly at the third day of exposure followed by a decrease at longer exposure times (Calisi et al., 2016). In contrast, Gomes et al. (2015) observed that MTs in *E. fetida* were not affected by Ag NPs or $AgNO_3$ even at higher exposure

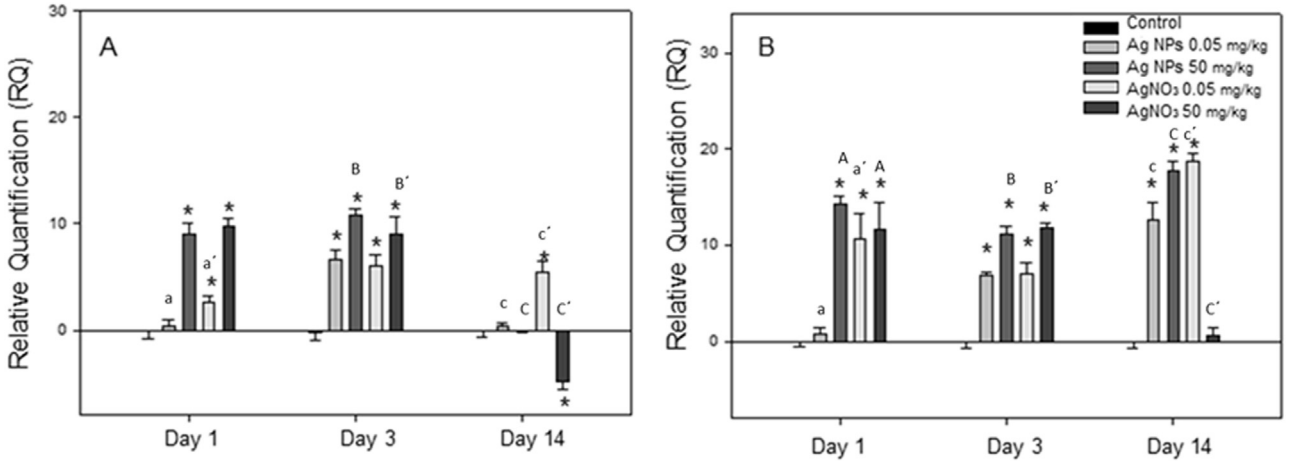

Fig. 5. Transcription levels of *mt* (A) and *cat* (B) in coelomocytes extruded from control earthworms and from earthworms exposed to Ag NPs and $AgNO_3$ (0.05 and 50 mg/kg) for 1, 3 and 14 days. Relative Quantification (RQ) means and standard deviations are shown. *Statistically significant differences with respect to the control group ($p < 0.001$). Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.001$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

concentrations than the ones used in the present work. These controversial results can be the effect of different concentrations and exposure times. In any case, it can be concluded that there is no full discrimination between the two Ag forms regarding MT levels.

Accordingly with Gomes et al. (2015) CAT activity in *E. fetida* was activated in short exposure periods (1 and 3 days) and inhibited after long periods of exposure to both Ag forms. This inhibition has been previously demonstrated in earthworms after exposure to metals by producing relevant quantities of superoxide anions (Irizar et al., 2014b). Recent studies evidence oxidative stress (with temporal changes) as a mechanism of toxicity after exposure to both silver forms in *E. fetida* earthworms (Hayashi et al., 2012, 2013; Tsyusko et al., 2012) and present results reinforce this idea. However, such studies showed that antioxidant responses to $AgNO_3$ started earlier than to Ag NPs, which could be related to oxidation time (quicker ion release) or a slower uptake of Ag NPs or due to a complexation with the soil matrix of the nanoform. Presently, CAT activity did not show a clear dissimilar patter between the two Ag forms.

The interaction of NPs or released ions with thiol groups of vital enzymes and proteins affects cellular processes and ultimately can lead to cell death (Hayashi et al., 2012; Levard et al., 2012). Cytotoxicity and trophic indexes were determined in coelomocytes extruded from organisms exposed to both Ag counterparts to assess cell viability. Coelomocytes are involved in eliminating foreign material by phagocytosis and encapsulation (amoebocytes) and they also synthesize and secret cytolitic components into the coelomic fluid (chloragocytes or eleocytes), causing lysis of non-self material (Bilej et al., 2010). Several studies have reported that the number of coelomocytes varies after exposure to metals as a result of changes in the permeability of the cell membrane that leads to diminished cell viability (Irizar et al., 2015b; Podolak et al., 2011). Similarly, Irizar et al. (2014a) found that *in vivo* and *in vitro* exposure to sublethal concentrations of metals (Pb, Ni, Cd, Cu) provoked a dose-dependant decrease in Neutral Red Uptake capacity due to damage in the coelomocytes membrane. Likewise, after *in vivo* exposure to Ag NPs and $AgNO_3$, the viability of coelomocytes decreased following a dose and time trend. Both silver forms could have released ions to soil pore water that would have entered trough the dorsal pores of the earthworms tegument (García-Velasco et al., 2016; Irizar et al., 2015a) and impact in the permeability of the cellular membrane (McShan et al., 2014) of coelomocytes, causing the observed cytotoxicity. The degree of Ag NPs solubilisation seems to be crucial to exert biological effects. Moreover, the toxicity of Ag NPs has been principally attributed to bioavailable Ag^+ ions (Van Aerle et al., 2013). However, it cannot be discarded in which form remained Ag NPs and $AgNO_3$ under present exposure conditions, in pore water as particulate form, soluble salts or insoluble Ag (nano)clusters or bound to soil particles conforming heteroaggregates. The response of the different subpopulations (amoebocytes and eleocytes) was dependant of the Ag form. In fact, according to coelomocytes viability and the trophic indexes, the exposure to Ag NPs (and to Ag^+ ions released from them) provoked a more marked toxicity than exposure to $AgNO_3$. This could be related with the target cell for each Ag form, hence Ag NPs enhanced the mortality of amoebocytes (increased the relative number of eleocytes), while $AgNO_3$ posed a decrease in eleocytes. Likewise, recent *in vitro* tests with coelomocytes demonstrated the selective intracellular accumulation of Ag NPs in the amoebocyte subpopulation and their role as scavengers of Ag NPs, effecting cytokine release and even death of the cell (Hayashi et al., 2012). Thus, a phagocytic uptake of Ag NPs may have occurred in amoebocytes, followed by intracellular particle oxidation which can produce cellular damage (Hayashi et al., 2012; Limbach et al., 2007). The intracellular accumulation of Ag NPs could act as Ag^+ source that is known as Trojan horse effect (Limbach et al., 2007). In contrast, studies with metal salts (*e.g.* $CdCl_2$) demonstrated eleocytes to be more sensitive than amoebocytes (Irizar et al., 2015b) and the same could happen with Ag salts ($AgNO_3$). Hence, both Ag forms caused cytotoxicity in coelomocytes but dissimilar sensitivities were recorded among subpopulations depending on the Ag form.

Apart from assessing metal detoxification (MT) and oxidative stress (CAT) in earthworm tissues, advances in molecular biology propelled the use of a new family of biomarkers based on the analysis of transcription levels of stress-related genes. In this framework, changes in the transcription levels of target genes such those encoding CAT or MT have been easily measured in earthworms subjected to Ag NPs and $AgNO_3$ (Hayashi et al., 2013; Tsyusko et al., 2012). In all these studies gene expression was measured in tissues whereas presently transcription levels were, for the first time, recorded in isolated coelomocytes extruded from exposed earthworms. According to the results obtained at biochemical level, *mt* transcription levels increased at days 1 and 3 followed by an inhibitory response after 14 days of exposure to the highest concentration. Equally, Tsyusko et al. (2012) pointed out that the highest number of significant changes in the levels of expression of *mt* in *E. fetida* exposed to both Ag NPs and $AgNO_3$ occurred at short exposure periods (up to 3 days) as can be expected for a early warning biomarker of metal exposure. It can be concluded that the transcription of *mt* is involved in short term homeostasis mechanisms for Ag exposure. Previous works dealing with *cat* expression in *E. fetida* showed different regulation patterns and temporal variation maybe due to a different bioavailability of the Ag in the media or related to the Ag NPs concentration and characterization (*i.e.* coating agent and size) (Hayashi et al., 2013; Tsyusko et al., 2012). Nevertheless, the dissimilar changes in eleocyte and amoebocyte number after Ag NPs and $AgNO_3$ exposure found in the present study should be taken into consideration when analyzing transcription level profiles of the whole coelomocyte population. In fact, the basal transcription level of each gene in each subpopulation might be different. Thus, subpopulation specific gene transcription profiles are found relevant for further studies including cell sorting techniques. However, the results obtained at transcription level in coelomocytes of earthworms exposed to Ag NPs and $AgNO_3$ were able to reflect responses at higher levels of biological complexity and thus, the utilization of immune cells to assess molecular level endpoints represents a relevant alternative for development of non-invasive biomarkers.

5. Conclusions

Despite the lack of effects in traditional endpoints (mortality and weight loss), Ag NPs and $AgNO_3$ posed changes at lower levels of biological complexity. Both Ag forms induced similar responses in most of the endpoints (significant changes in the metal detoxification mechanism and in the antioxidant response system). In contrast, at cellular level cytotoxicity was higher after exposure to Ag NPs but, dissimilar sensitivities were recorded among coelomocytes subpopulations depending on the Ag form, suggesting a different mode of action of nanoparticulate/salt/free ionic Ag depending on the target cell.

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