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Effects of soil and dietary exposures to Ag nanoparticles and $AgNO_3$ in the terrestrial isopod *Porcellionides pruinosus*

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ABSTRACT

The effects of Ag-NPs and $AgNO_3$ on the isopod *Porcellionides pruinosus* were determined upon soil and dietary exposures. Isopods avoided Ag in soil, with EC50 values of ~16.0 and 14.0 mg Ag/kg for Ag-NPs and $AgNO_3$, respectively. Feeding inhibition tests in soil showed EC50s for effects on consumption ratio of 127 and 56.7 mg Ag/kg, respectively. Although similar EC50s for effects on biomass were observed for nanoparticulate and ionic Ag (114 and 120 mg Ag/kg dry soil, respectively), at higher concentrations greater biomass loss was found for $AgNO_3$. Upon dietary exposure, $AgNO_3$ was more toxic, with EC50 for effects on biomass change being >1500 and 233 mg Ag/kg for Ag-NPs and $AgNO_3$, respectively. The difference in toxicity between Ag-NPs and $AgNO_3$ could not be explained from Ag body concentrations. This suggests that the relation between toxicity and bioavailability of Ag-NPs differs from that of ionic Ag in soils.

1. Introduction

Silver nanoparticles (Ag-NPs) are widely used in the nanotechnology industry and consumer products, especially due to their bactericidal properties. The production of Ag-NPs has increased in the last years, and it is one of the most produced NPs on the market (Meyer et al., 2009).

Due to the release from NP-containing products, Ag-NPs may enter the aquatic environment and reach the soil through the land application of treated sewage sludge or biosolids (Gottschalk et al., 2009; Kaegi et al., 2011). In sewage sludge, the concentration was estimated to reach ~1.7 mg Ag/kg in Europe (Gottschalk et al., 2009). In biosolids-amended soils, however, much lower concentrations are expected, although the continuous input of biosolids to agricultural land may lead to increasing Ag-NP concentrations over time. For example, an increase rate of 36 μg Ag/kg/y is estimated for agricultural land through sludge application in the United Kingdom (Whiteley et al., 2013). Modeled Ag-NP concentrations in soils exclusively treated with sludge ranged from ~10 to 100 μg/kg in Europe (Gottschalk et al., 2013). However, in sludge-free soils, estimated Ag-NP concentrations were 0.1–1 μg/kg (Gottschalk et al., 2013).

Toxicity studies on Ag-NPs in soil have shown that responses may occur at low levels of exposure. In a field experiment, effects of Ag-NPs on plants and microbial processes were found at concentrations as low as 0.14 mg Ag/kg (Colman et al., 2013). And avoidance behavior of earthworms, measured as EC50, has been reported at ~4–8 mg Ag/kg in natural soil (Shoults-Wilson et al., 2011b).

Up to date, dietary toxicity of NPs to isopods has previously been tested for Cu-NPs (Golobic et al., 2012), TiO_2-NPs (Valant et al., 2012), ZnO-NPs (Pipan-Tkalec et al., 2010), and Ag-NPs (Tkalec et al., 2011). Only two studies have evaluated the effects of soil exposure on isopods for ZnO and CeO_2-NPs (Tourinho et al., 2013, 2015). Previous studies with the isopod *Porcellionides pruinosus* have shown that different routes of exposure (i.e. food and soil) need to be evaluated to properly assess the effects of contaminants on isopods (Sousa et al., 2000; Vink et al., 1995).

The objective of this study was to evaluate the toxicity of Ag-NPs and ionic Ag to the terrestrial isopod *P. pruinosus*, using soil and food as exposure routes. For this purpose, avoidance behavior and feeding inhibition were evaluated in natural soil spiked with Ag-NPs and ionic Ag ($AgNO_3$). Additionally, a feeding inhibition test

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was conducted with Ag-dosed food, in order to assess the effects of Ag-NPs and $AgNO_3$ upon dietary exposure and compare with soil exposure.

2. Methodology

2.1. Test organisms

Specimens of the isopod *P. pruinosus* were collected from a horse manure heap in an uncontaminated field (Coimbra, Portugal). The animals were kept in the lab at 20 ± 2 °C and a 16/8 h photoperiod for at least one month before use in the tests. For the toxicity tests, healthy adult males and non-gravid females (>15–25 mg) were used. Animals without antenna were not used, as their chemoreceptor organs are located in the apical organ of their second antenna, which can perceive chemicals and test stimuli.

2.2. Exposure media and test chemical

Lufa 2.2 soil (LUFA-Speyer 2.2, Sp 2121, LUFA Speyer, Speyer, Germany) and alder (*Alnus glutinosa*) leaves were used for the exposures. Lufa 2.2 is a loamy sand soil with pH (0.01 M $CaCl_2$) 5.5 ± 0.2, water holding capacity (WHC) 41.8 ± 3.0%, 1.77 ± 0.2% organic C, 0.17 ± 0.02% nitrogen, 7.3 ± 1.2% clay; 13.8 ± 2.7% silt and 78.9 ± 3.5% sand. Alder leaves were collected from an uncontaminated area in Coimbra (Portugal) and stored at room temperature.

Soil and leaves were spiked with Ag-NPs (AMEPOX, 3–8 nm, alkane coating) dispersed in pure water at 1000 mg/L or $AgNO_3$ (Sigma–Aldrich, 99% purity) also dissolved in water. Result of Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) analyses of the Ag-NPs can be found in Figs. SI-1 and SI-2, respectively. Soils were moistened to 45% of the WHC, and left to equilibrate for one day before use in the toxicity tests.

2.3. Avoidance behavior test

Lufa 2.2 soil was spiked with both Ag forms at nominal concentrations of 1, 5, 10, 50, and 100 mg Ag/kg, while for Ag-NPs also 500 mg Ag/kg was tested. Plastic boxes (135 × 85 mm) were divided in two compartments using a removable plastic split, where 40 g of moist Ag-spiked soil were added in one side and 40 g unspiked soil were added in the opposite side of the box (for details, see Loureiro et al. (2005)). The split was removed and 3 isopods were introduced. Three replicates were used for each concentration. Avoidance behavior was visually observed after 2 h to check if an immediate response could be seen. After 48 h, the split was reintroduced and the number of animals counted in each compartment (spiked or unspiked soil). The test also included a control having unspiked soil in both compartments.

2.4. Feeding inhibition test – soil exposure

Lufa 2.2 soil was spiked at nominal concentrations of 50, 100, 200, 400, and 800 mg Ag/kg for Ag-NPs and 12.5, 25, 50, 100, and 200 mg Ag/kg for $AgNO_3$. These concentrations were based on a preliminary study, in which $AgNO_3$ showed high mortality at concentrations above 200 mg Ag/kg.

Isopods were weighted and placed individually in plastic boxes (⌀ 65 mm), containing 20 g of moist soil. Five replicates were used for each concentration and control (unspiked soil). The animals were fed *ad libitum* with pieces of alder leaves that were dried at 50 °C and weighted. After 14 days, isopods were left for 1 day without food to empty their guts. Isopods and remaining food were weighted.

2.5. Feeding inhibition test – dietary exposure

For food exposure, alder leaves were cut in pieces of ~50 mg (dry weight). Solutions of Ag-NPs or $AgNO_3$ at different concentrations were topically added to the leaves' surface with a micropipette. Solution concentrations were chosen in order to add the same volume of solution (400 μL) to each leaf portion. Half of this volume was applied on each side of the leaves to guarantee a more homogeneous distribution of Ag on the surface. A control with ultrapure water was also included. Leaves were dried at room temperature for one day before being offered to the isopods as food (*ad libitum*). The test vessel was composed of two plastic boxes (⌀ 85 mm) placed inside each other (Loureiro et al., 2006). The inner box with a net in the bottom was used to easily collect the faeces and avoid coprophagy. The outer box had a bottom of plaster of Paris and was used to maintain high air humidity. Individual isopods and food were placed in the chambers, with 10 replicates per concentration. After 14 days exposure, the fresh weight of isopods and dry weight of remaining food and faeces were measured.

2.6. Chemical analysis

Soil pH was measured in 0.01 M $CaCl_2$ extracts of freshly spiked soils, in accordance to ISO guideline 10390 (ISO, 1994).

For total Ag analysis, single replicate samples of approximately 130 mg soil or 30 mg leaf material were dried overnight at 50 °C. The samples were then digested in 2 mL of a mixture of concentrated HCl (J.T. Baker, purity 37%) and HNO_3 (J.T. Baker, purity 70%) (4:1, v/v) for 7 h in an oven (CEM MDS 81-D) at 140 °C, using tightly closed Teflon containers. After digestion, the samples were taken up in 10 mL of demineralized water and analyzed by flame atomic absorption spectrometry (AAS) (Perkin–Elmer AAnalyst 100). Certified reference material (ISE sample 989 of River Clay from Wageningen, The Netherlands) was used to ensure the accuracy of the analytical procedure. Ag concentration in the reference material (mean ± SE; n = 2) was 125 ± 1.4% of the certified value.

Total Ag concentrations were also determined in the isopods. After freeze-drying, three isopods from each treatment were individually weighted and digested in 300 μL of a mixture of HNO_3:$HClO_4$ (7:1, v/v, J.T. Baker, ultrapure). The samples were evaporated to dryness and the residues were taken up in 1 mL 1 M HCl. Silver content was determined by graphite furnace AAS (Perkin–Elmer 5100 PC).

2.7. TEM images

Transmission Electron Microscopy (TEM) analyses were performed on unspiked soil and on soil spiked with Ag-NPs and $AgNO_3$ at 800 and 200 mg Ag/kg, respectively. Approximately 10 mg of soil were dispersed in 10 mL deionized water, and sonicated in an ultrasonic bath for 30 s. Then, 20 μl of the dispersion was suspended on a carbon coated Cu TEM grid. TEM was carried out on a 200 kV analytical JEOL 2010 instrument with an Oxford Instruments EDX detector. TEM micrographs were taken from several regions with small grains. Large grains are not electron transparent and were excluded from this analysis.

2.8. Data analysis

Avoidance response (%) was calculated as Loureiro et al. (2005):

$$A = (C - T)/N * 100$$

where C is the number of animals in control soil, T is the number of animals in spiked soil and N is the total number of animals recovered from the soil. The median effect concentration (EC50) was

calculated using a two-parameter logistic curve.

For the feeding inhibition test, the feeding parameters were calculated as:

$$Cr = \left(W_{Li} - W_{Lf}\right) \Big/ W_{isop}$$

$$Ar = \left(\left(W_{Li} - W_{Lf}\right) - F\right) \Big/ W_{isop}$$

$$Ae = \left((W_{Li} - W_{Lf}) - F\right) \Big/ (W_{Li} - W_{Lf}) * 100$$

$$Eg = F / W_{isop}$$

$$Bc = \left(\left(W_{isopf} - W_{isop}\right) \Big/ W_{isop}\right) * 100$$

where, W_{Li}—initial leaf weight (mg d.w.); W_{Lf}—final leaf weight (mg d.w.); W_{isop}—initial isopod weight (mg f.w.); W_{isopf}—final isopod weight (mg f.w.); F—faeces (mg d.w.); Cr—Consumption ratio (mg leaf/mg isopod); Ar—assimilation ratio (mg leaf/mg isopod); Ae—assimilation efficiency (%); Eg—egestion ratio (mg faeces/mg isopod); Bc—biomass change (%).

For the feeding inhibition test in soil, consumption ratio was analyzed by one-way analysis of variance (ANOVA) after log-transformation. The median effect concentration (EC50) for the consumption ratio (mg food/mg isopod) and biomass change (% of fresh weight) was calculated with a four-parameter logistic regression. The Bioaccumulation factor (BAF) was calculated as the ratio between Ag concentration in the isopods and total Ag concentration in soil. For the feeding inhibition test upon dietary exposure, the feeding parameters were log transformed to achieve normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test) and analyzed by one-way ANOVA followed by Dunnett's post hoc test. If even after log transformation data failed normality or homoscedasticity tests, a Kruskal–Wallis one-way analysis of variance on ranks was conducted. When significant differences were found, a Dunn's post-hoc test was performed.

The relation between biomass change (%) and Ag body concentration in isopods was tested by Spearman correlation analysis, and the relation between Ag body concentration in isopods and Ag concentrations in soil/food by regression analysis. Normality of the residuals was tested by Kolmogorov–Smirnov test and homoscedasticity was graphically analyzed by plotting standardized residuals versus predicted values. Data was squared root transformed when necessary. Analysis of covariance (ANCOVA) was conducted to compare the slopes obtained in the regression analysis, with Ag form as independent variable, Ag body concentration as dependent variable and Ag concentration in soil/food as covariant. The homogeneity of slopes was tested prior to the analysis ($p < 0.05$) and normality and homoscedasticity were tested with Kolmogorov–Smirnov and Levene's tests, respectively. For dietary exposure, ANCOVA analysis could not be performed, since assumptions were violated even after data linearization.

3. Results

3.1. Ag analysis in soil and food

Measured total Ag concentrations in the soils of the avoidance behavior and feeding inhibition tests are shown in Tables SI-1 and SI-2, respectively. In general, Ag recovery was satisfactory, ranging between 75 and 125% of the nominal concentrations (Table SI-1 and SI-2). Recovery was lower at 800 mg Ag/kg (55%) for Ag-NPs and at 50 and 100 mg Ag/kg (67–68%) for $AgNO_3$ in the feeding inhibition test, and higher at 1 mg/kg (310–320%) for both Ag forms and at 5 and 10 mg Ag/kg (170–187%) for $AgNO_3$ in the avoidance test.

Measured Ag concentrations in the leaves are shown in Table SI-3. In most treatments, recovery was good for both Ag forms, ranging from 62 to 114%. However, at the highest nominal concentration of 3000 mg Ag/kg, recovery was 50 and 39% for Ag-NPs and $AgNO_3$, respectively.

All effect concentrations reported in this paper were calculated based on measured Ag concentrations in soil or food.

3.2. Soil properties

Soil pH showed little difference among Ag-NPs spiked soils, ranging from 5.52 to 5.81 (Table SI-2). For ionic Ag spiked soils, a slight decrease in soil pH was observed with increasing Ag concentration, from 5.57 at 12.5 mg Ag/kg to 5.33 at 200 mg Ag/kg (Table SI-2).

TEM of unspiked Lufa 2.2 soil showed a low background of Ag (0.1–0.2%) (Fig. SI-3A). In soil spiked with Ag-NPs at 800 mg Ag/kg, clusters of Ag particles could be found in many areas (Fig. SI–3B), which closely resemble the original Ag particles. A small number of larger particles could also be detected, ranging from 20 to 50 nm (Fig. SI–3C). At higher magnification, it was possible to identify individual Ag particles, with particle sizes ranging from 5 to 8 nm (Fig. SI-3D). TEM images of Lufa soil spiked with $AgNO_3$ can be found in Fig. SI-4, where no particles could be pinpointed.

3.3. Avoidance behavior test

No mortality of isopods was observed during the avoidance behavior test. The isopods were able to avoid both Ag-NPs and ionic Ag in soil (Fig. 1; Table 1). Based on the overlap of 95% confidence intervals, no difference in EC50 was observed between both Ag forms. A limit value of >80% of organisms located in the control soil is considered to indicate impairment of the habitat function of soils (Hund-Rinke and Wiechering, 2001). In the present study, >80% of the isopods were found in the control soil at 36 and 18 mg Ag/kg dry soil for Ag-NPs and ionic Ag, respectively (Fig. 1).

3.4. Feeding inhibition test – soil exposure

No mortality was observed in isopods exposed to Ag-NPs, while one out of five animals died in the ionic Ag exposure at 100 mg Ag/

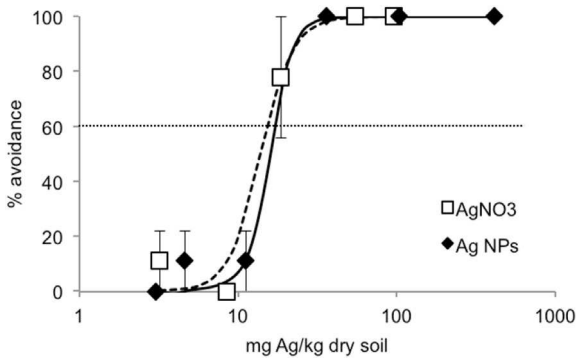

Fig. 1. Percentage (mean ± SE; n = 3) of isopods (*Porcellionides pruinosus*) in control soil after 48 h in the avoidance behavior test with Ag-NPs and ionic Ag ($AgNO_3$) on Lufa 2.2 soil. Solid (Ag-NPs) and dash ($AgNO_3$) lines represent the fit obtained with a 2-parameter logistic dose-response model. The dotted line represents the criterion for habitat function, where >80% of the organisms were located in the control soil (equal to 60% avoidance).

Table 1
LC50 for effects for Ag NPs and $AgNO_3$ on the survival, EC50 for effects on consumption ratio, biomass change and avoidance behavior of the isopod *Porcellionides pruinosus*. LC50 and EC50 values for effects on consumption ratio and biomass were obtained after 14 d exposure to Ag-dosed Lufa 2.2 soil or alder leaves. EC50 values for avoidance behavior were calculated from the 48-h avoidance behavior test in Lufa 2.2 soil. 95% Confidence intervals are presented in between brackets.

	Soil exposure				Dietary exposure
	LC50	EC50 consumption ratio	EC50 biomass	EC50 avoidance behavior	EC50 biomass
Ag NPs	>455	127 (56.4–200)	114 –	15.8 (0.24–31.4)	>1500
$AgNO_3$	396[a] (235–745)	56.7 (8.33–105)	120 –	13.9 (10.1–17.7)	233 –

[a] Based on nominal concentrations.

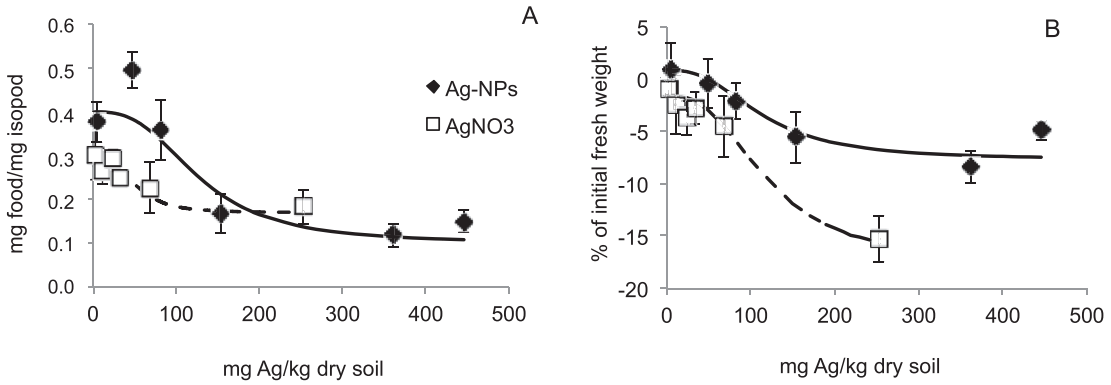

Fig. 2. Effects of Ag NPs and ionic Ag ($AgNO_3$) on the consumption ratio (mean ± SE; n = 5) (A) and biomass change (mean ± SE; n = 5) (B) of the isopod *Porcellionides pruinosus* after 14 days exposure in Lufa 2.2 soil. Solid (Ag NPs) and dash ($AgNO_3$) lines represent the fit obtained with a 4-parameter logistic dose-response model.

kg dry soil. Consumption ratio (mean ± SE; n = 5) was 0.38 ± 0.05 and 0.30 ± 0.06 mg food/mg isopod in control animals for the tests with Ag-NPs and $AgNO_3$, respectively (Fig. 2A). Consumption ratio was significantly decreased in isopods exposed at 153 and 252 mg Ag/kg dry soil for Ag-NPs and ionic Ag, respectively (One-way ANOVA, Dunnett's posthoc test). EC50s for effects on consumption ratio can be found in Table 1.

Biomass change showed a dose-related decrease in isopods exposed to both Ag-NPs and ionic Ag (Fig. 2B). Even though EC50s for both Ag forms were similar (Table 1), biomass was strongly reduced by ionic Ag at higher concentrations. Biomass was reduced up to 8.2% at 361 mg Ag/kg for Ag-NPs, while for ionic Ag, biomass reduction was up to 15.2% at 251 mg Ag/kg. In a pilot test, ionic Ag caused high isopod mortality at 400 and 800 mg Ag/kg dry soil, indicating it is more toxic than Ag-NPs.

Total Ag body concentration in isopods dose-related increased for both Ag forms (Fig. 3). A significant linear relationship was found for Ag-NPs (r^2 = 0.77, p = 0.00) and $AgNO_3$ (r^2 = 0.72, p = 0.00). No significant difference in the regression slopes was observed between Ag-NPs and $AgNO_3$ (ANCOVA, F = 1.62, p > 0.05), but soil concentration significantly affected Ag body concentration (ANCOVA, F = 81.5, p = 0.00). Bioaccumulation factors (BAF) were similar for the two Ag forms and tended to decline with increasing exposure level (Table SI-2).

To link toxicity to Ag body concentration, effects on biomass were related to Ag in body using a correlation analysis (Fig. 4). No significant relationship was found for Ag-NPs (Spearman correlation, r = −0.16, p = 0.51), while for ionic Ag a weak but significant relation was found (Spearman correlation, r = −0.65, p = 0.00).

3.5. Feeding inhibition test - dietary exposure

No mortality was observed in control animals, while mortality rate was 20% (2 out of 10 isopods) for Ag-NP exposure at 114 mg Ag/

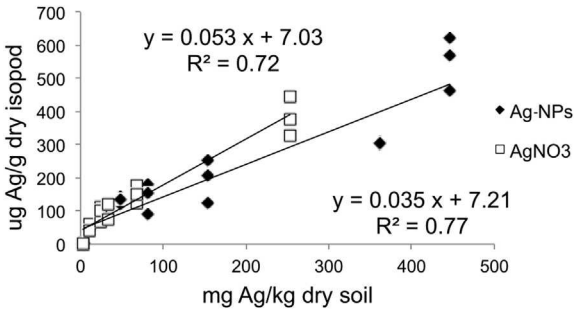

Fig. 3. Linear regression between Ag body concentrations (μg Ag/g dry body weight) and total Ag concentrations in Lufa 2.2 soil (mg Ag/kg dw) in the isopod *Porcellionides pruinosus* after 14 days exposure to Ag-NPs (◆) and ionic Ag as $AgNO_3$ (□).

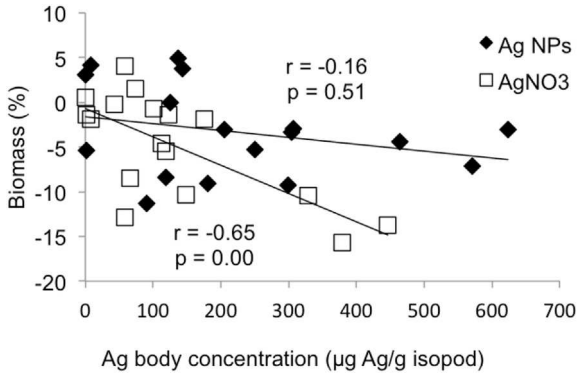

Fig. 4. Biomass change (%) as a function of Ag body concentration (ug Ag/g dry body weight) in the isopod *Porcellionides pruinosus* exposed for 14 days to Ag NPs (◆) and ionic Ag (□) in Lufa 2.2 soil (n = 3 for each exposure concentration in soil). Spearman correlation coefficient (r) and p-values are given for Ag-NPs and $AgNO_3$.

kg dry food. For $AgNO_3$, mortality rate was 60% (6 out of 10 isopods) at 279 and 785 mg Ag/kg dry food, and 40% at 1159 mg Ag/kg dry food.

Consumption ratio in the control (0.82 mg food/mg isopod) was significantly higher than for both Ag-NP (One-way ANOVA, $F = 6.76$, $p < 0.05$) and $AgNO_3$ exposure ($F = 7.13$, $p < 0.05$) (Fig. 5A). Upon Ag-NP exposure, consumption ratio ranged from 0.35 to 0.49 mg food/mg isopod and was significantly lower than the control in all treatments (Dunnett's test, $p < 0.05$). Upon $AgNO_3$ exposure, consumption ratio varied from 0.22 to 0.48 mg food/mg isopod, and was significantly different from the control for all treatments, except for 62 mg Ag/kg dry food (Dunnett's test, $p < 0.05$). Assimilation ratio in control animals was 0.45 mg food/mg isopod, while it ranged between 0.25–0.42 and 0.18–0.38 mg food/mg isopod for the Ag-NP and $AgNO_3$ exposures, respectively (Fig. 5B). Assimilation ratio decreased significantly in isopods exposed to Ag-NPs (Kruskal–Wallis one-way ANOVA on ranks, $H = 15.78$, $p < 0.05$) and $AgNO_3$ ($H = 12.98$, $p < 0.05$), with significant differences at 29 mg Ag/kg dry food for Ag-NPs, and at 22 and 279 mg Ag/kg dry food for $AgNO_3$ (Dunn's test, $p < 0.05$). Assimilation efficiency increased with increasing concentration for both Ag exposures (Fig. 5C). However, significant differences were only found for $AgNO_3$ (Kruskal–Wallis one-way ANOVA on ranks, $H = 13.02$, $p < 0.05$), but not for Ag-NPs ($H = 9.54$, $p > 0.05$). Assimilation efficiency was significantly higher at the highest concentration of $AgNO_3$ (92%) compared with the control (71%) (Dunn's test, $p < 0.05$). In control animals, egestion ratio (0.30 mg faeces/mg isopod) was significantly higher than for Ag-NP (0.05–0.12 mg faeces/mg isopod) (Kruskal–Wallis one-way ANOVA, $H = 17.21$, $p < 0.05$) and $AgNO_3$ exposures (0.05–0.13 mg faeces/mg isopod) ($H = 20.34$, $p < 0.05$) (Fig. 5D). Egestion ratio was significantly decreased at concentrations ≥218 mg Ag/kg dry food for Ag-NPs, and at 279 and 1159 mg Ag/kg dry food for $AgNO_3$ (Dunn's test, $p < 0.05$).

Biomass increased 3.16% in control animals after 14 days. Ag-NP exposure decreased biomass from 0.48 to −1.85%, but this decrease was not dose-related to Ag concentration in food (Fig. 5E). No significant difference was found between Ag-NP and control treatments (Kruskal–Wallis one-way ANOVA, $H = 8.32$, $p > 0.05$). Upon $AgNO_3$ exposure, biomass significantly decreased with increasing Ag concentration in food ($H = 14.59$, $p < 0.05$). A significant difference was observed at the highest concentration (Dunn's test, $p < 0.05$), with mean biomass change of −5.72%. For $AgNO_3$ exposures, EC50 was 233 mg Ag/kg (Fig. 5E).

Ag body concentration tended to increase with increasing Ag concentration in food (Fig. 6). A steeper slope was observed for $AgNO_3$ in comparison to Ag-NPs. No significant relationship was found between biomass change and Ag body concentrations upon dietary exposure to both Ag forms (data not shown).

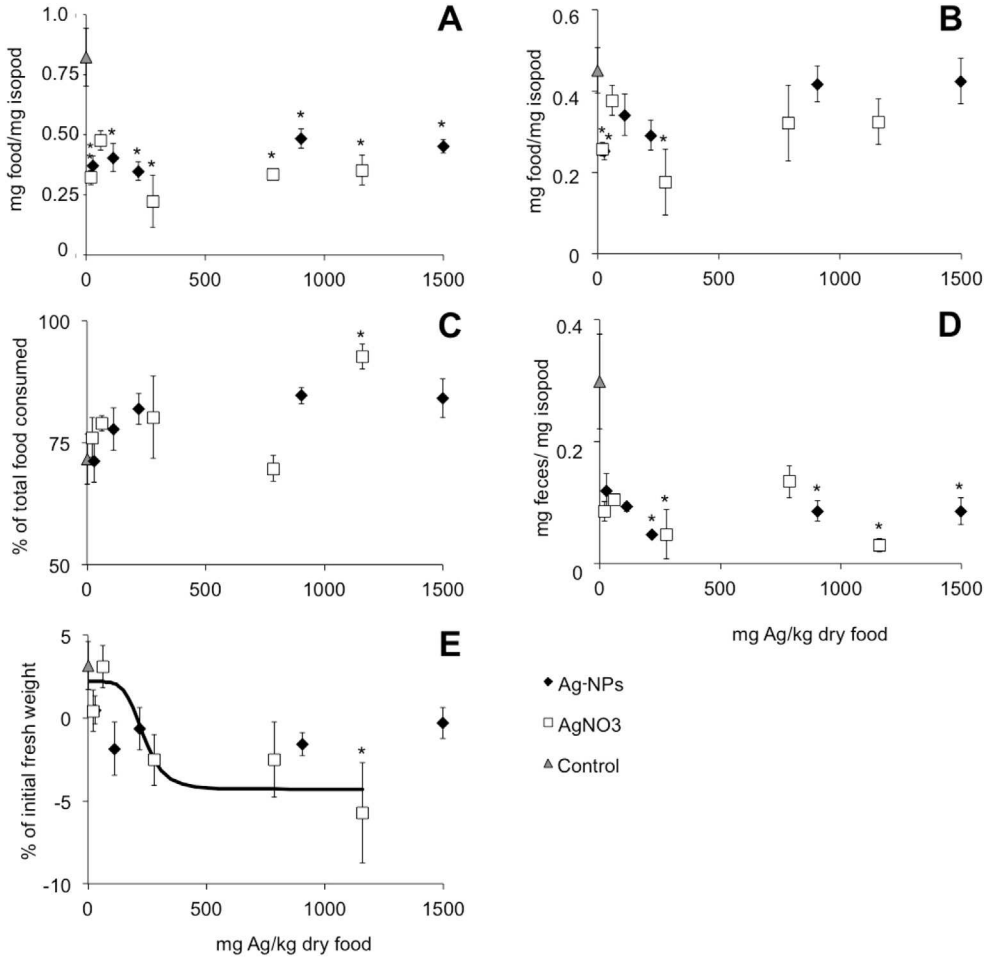

Fig. 5. Effects on consumption ratio (a), assimilation ratio (b), assimilation efficiency (c), egestion ratio (d), and biomass change (e) of isopods (*Porcellionides pruinosus*) exposed for 14 days to Ag-NP and $AgNO_3$ dosed food (mg Ag/kg dry food). Line represents the fit obtained with a 4-parameter logistic dose-response model for effects of $AgNO_3$ on isopod biomass change.

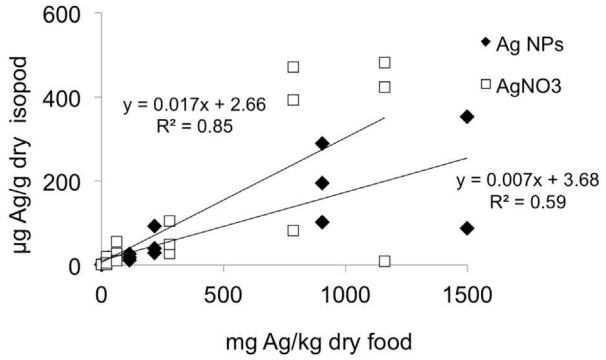

Fig. 6. Ag body concentrations (µg Ag/g dry body weight) in the isopod *Porcellionides pruinosus* as a function of total measured Ag concentrations in food (mg Ag/kg dw) after 14 days exposure to Ag-NPs (◆) and ionic Ag as $AgNO_3$ (□). Lines represent the linear relationship between mean Ag body concentration and mean measured Ag concentration in soil.

4. Discussion

Here, isopods were exposed to Ag-NPs and $AgNO_3$ via soil and food. When comparing the EC50 for effects on biomass change (Table 1), higher toxicity of both Ag-NPs and $AgNO_3$ were found following soil exposures. These findings are in agreement with the study of Vink et al. (1995), who found higher LC50s for the same species exposed to three pesticides via food in comparison to substrate (e.g., sand).

In our study, the slopes of the relation between Ag body concentration and exposure concentration were 5 and 3 times higher for soil exposure than for dietary exposure for Ag-NPs and $AgNO_3$, respectively. Similarly, Sousa et al. (2000) observed that lindane assimilation rate in *P. pruinosus* was up to 20 times higher in soil exposures. The lower bioavailability and toxicity upon dietary exposure was explained by the higher organic matter content of food, resulting in stronger binding of contaminants in comparison to soil (Sousa et al., 2000; Vijver et al., 2006; Vink et al., 1995).

4.1. Ag availability in soil

The toxicity of NPs will depend not only on their properties, but also on the processes occurring in soils, such as aggregation/agglomeration and dissolution. In the present study, Ag-NPs were found as small aggregates of >50 nm and as single particles (5–8 nm) when spiked into the natural Lufa 2.2 soil (Fig. SI-3).

Aggregation may affect dissolution rates, as dissolution was found to be slower for larger aggregates (Coutris et al., 2012). Dissolution of Ag ions from Ag-NPs occurs through oxidation processes, which will depend on reactions on the particle surface (Batley et al., 2012; Shoults-Wilson et al., 2011a). However, low levels of oxidized Ag are normally reported in the literature (Cornelis et al., 2012, 2013; Coutris et al., 2012; Shoults-Wilson et al., 2011a; Waalewijn-Kool et al., 2014). Ag-NPs may bind to soil particles (Coutris et al., 2012), especially to clays resulting in low Ag concentrations in pore water (Cornelis et al., 2012).

In agreement, only low Ag levels were found in the pore water of Lufa 2.2 soil spiked with Ag-NPs and $AgNO_3$ (Waalewijn-Kool et al., 2014). The authors found that Ag pore water concentration was less than 1.5% of the total Ag concentration in soil. In the present study, similar conditions and the same Ag-NPs were used as in the study by Waalewijn-Kool et al. (2014). Thus, Ag porewater concentrations in our study are also expected to be rather low in both Ag-NP and $AgNO_3$ spiked soils.

4.2. Avoidance behavior

The EC50 values for Ag-NPs and ionic Ag indicated that isopods were able to detect and avoid Ag at relatively low soil concentrations, independent of the Ag form. As low levels of freely dissolved Ag are expected in soil pore water, we may conclude that not only Ag^+ ions but also the nanoparticles were responsible for the avoidance behavior of the isopods.

Similar high sensitivity of avoidance responses was found for the earthworm *Eisenia fetida* with EC50s of 4.80, 8.74, and 6.06 mg Ag/kg dry soil for 10 nm Ag-NPs, 30–50 nm Ag-NPs, and $AgNO_3$, respectively (Shoults-Wilson et al., 2011b). Avoidance of Ag-NPs was related to nanosized Ag, since the test duration (48 h) was not long enough to expect much dissolution of Ag^+ ions from the NPs (Shoults-Wilson et al., 2011b). Moreover, these authors observed an immediate avoidance of $AgNO_3$ spiked soil (after 2 h), but not of Ag-NP spiked soil, that could be due to the faster perception of Ag^+ ions by the earthworms. In our study, no avoidance of ionic Ag by the isopods was observed after 2 h of exposure, which could be explained from differences in exposure routes between isopods and earthworms. Earthworms not only have a soft body, but also live in close contact to soil, being dermally exposed to soil pore water (Van Gestel and Van Straalen, 1994). Isopods, on the other hand, have a hard body (cuticle), being less exposed to the dissolved ions in pore water (Van Gestel and Van Straalen, 1994).

4.3. Feeding inhibition – soil exposure

Ag-NPs and ionic Ag exposures decreased food consumption and biomass in isopods exposed via soil for 14 days. At concentrations up to 100 mg Ag/kg in soil, biomass decreased in the same dose-related manner for both Ag forms, leading to these similar EC50 values (Fig. 2B). However, at the higher exposure concentrations, ionic Ag showed to be much more toxic. A drastic decrease in the biomass was observed in isopods exposed to ionic Ag at >100 mg Ag/kg dry soil, while effects seemed to level off in isopods exposed to Ag-NPs up to ~500 mg/kg dry soil. Moreover, a preliminary test with ionic Ag (concentrations up to 800 mg Ag/kg dry soil) resulted in high mortality, with an LC50 of 396 mg Ag/kg dry soil (nominal concentration, data not shown). For Ag-NPs, no mortality was observed in the isopods exposed up to ~500 mg Ag/kg dry soil. Our results confirm the difference in toxicity between Ag-NPs and ionic Ag. For instance, effects on biomass could not be related to Ag body concentration for Ag-NPs, while for $AgNO_3$ biomass was negatively related with Ag body concentration.

Nevertheless, the higher toxicity observed for ionic Ag could not be explained from Ag bioaccumulation as no difference in Ag body concentration was found between Ag-NPs and $AgNO_3$ when comparing the regression slopes. A lack in relationship between nanoparticle toxicity and body concentration was also observed in other studies with different nanoparticles. ZnO-NP toxicity, for instance, could not be directly related to Zn body concentration in earthworms (Heggelund et al., 2013) and isopods (Tourinho et al., 2013).

Still, it is unclear whether toxicity in isopods exposed to Ag-NPs is caused only by Ag ions dissolved from Ag-NPs inside the body or by a combination of nanosized and ionic Ag. Dissolved Ag from NPs was responsible for toxicity in the earthworms *E. fetida* and *E. andrei* (Schlich et al., 2013; Tsyusko et al., 2012), the collembolan *F. candida* (Waalewijn-Kool et al., 2013), and the nematode *Caenorhabditis elegans* (Meyer et al., 2010). Even though a particle effect was suggested, toxicity still was mainly related to ionic Ag for the potworm *Enchytraeus albidus* (Gomes et al., 2013) and the earthworm *E. fetida* (Gomes et al., 2015).

4.4. Feeding inhibition - dietary exposure

Isopods were able to detect and avoid food dosed with both Ag-NPs and ionic Ag, by decreasing food consumption. Avoidance of highly contaminated food has been observed in *P. pruinosus*, and considered to be metal-specific (Loureiro et al., 2006). EC50s for the effect on consumption ratio were 10.3 and 11.1 mg/g dry food for Cu and Zn, respectively, while no decrease in food consumption was observed in isopods exposed to Cd and Pb (Loureiro et al., 2006). Effects on food consumption have been observed for the isopod *Porcellio scaber* exposed to food dosed with Zn (Bibič et al., 1997; Donker et al., 1996; Drobne and Hopkin, 1995; Zidar et al., 2003) and Cu (Farkas et al., 1996), and for the isopod *Oniscus asellus* exposed to Co (Drobne and Hopkin, 1994). In contrast to the present study, no decrease in food consumption was observed for the isopod *P. scaber* exposed for 14 days to Ag-NP dosed food (up to 5000 μg Ag/g dry food) (Tkalec et al., 2011). Probably, the avoidance of Ag-dosed food differs between isopod species, while it may also depend on the type of food, the way of spiking the food and the type of NPs (e.g. coating). The authors used hazelnut leaves as food and Ag-NPs with a diameter size between 30 and 200 nm.

The avoidance of Ag-dosed food led to a decrease in assimilation and egestion ratios compared to the control, even though it did not decrease in a dose-related manner. Assimilation efficiency tended to increase with increasing Ag concentration in food. Isopods can increase assimilation efficiency by increasing the residence time of the food in the digestive tract, as a consequence of low quality or contaminated food (Drobne and Hopkin, 1994, 1995). Overall, inhibition of feeding activity was observed when isopods were exposed via food to both Ag-NPs and $AgNO_3$.

No significant decrease in biomass was observed in isopods exposed to Ag-NPs up to ~1500 mg Ag/kg dry food. In agreement, no effect of Ag-NPs on weight change was observed in the isopod *P. scaber* when exposed up to 5000 mg/kg for 14 days (Tkalec et al., 2011). Nevertheless, not only did biomass significantly decrease, biomass loss was also higher in isopods exposed to $AgNO_3$-dosed food when compared to Ag-NPs. Thus, toxic effects on biomass could only be observed for ionic Ag, but not for AgNPs, when exposed via food.

We used an indirect exposure by topically applying Ag as solution on the food. Indirect exposure was shown to lead to lower assimilation of Au-NPs in comparison to direct exposure (Judy et al., 2012; Unrine et al., 2012). One possible reason for that is the aggregation of NPs after the solution has dried on the leaf surface, decreasing NP bioavailability (Judy et al., 2012). If Ag-NP aggregation also increased due to the spiking procedure in our study, it could explain the slightly lower slope of the regression line for Ag body concentration in Ag-NP exposure.

5. Conclusions

Ag-NPs and $AgNO_3$ affected the avoidance behavior and feeding activity in isopods exposed via soil and food. The isopod *P. pruinosus* can avoid low concentrations of Ag in soil, independent of the Ag form (i.e., nanosized or ionic Ag). Still, these concentrations were around two-fold higher than predicted Ag concentrations in soils amended with sewage sludge.

In the feeding trials for both soil and dietary exposures, Ag-NPs were found generally to be less toxic than $AgNO_3$. Following soil exposure, ionic Ag caused greater biomass losses and mortality, while Ag-NPs caused no mortality and had less effect on biomass. Ag body concentrations failed to explain these differences in toxicity, since Ag similar levels were found in isopods exposed to Ag-NPs and ionic Ag. In agreement, upon dietary exposure, higher toxicity was found for ionic Ag, whereas no effects on the biomass could be observed in the Ag-NP treatments. These differences in toxicity between the two Ag forms, which need further investigation, may be key to an appropriate risk management of Ag-NPs in terrestrial environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.05.044.

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