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Protective effect of *N*-acetylcysteine on the toxicity of silver nanoparticles: Bioavailability and toxicokinetics in *Enchytraeus crypticus*

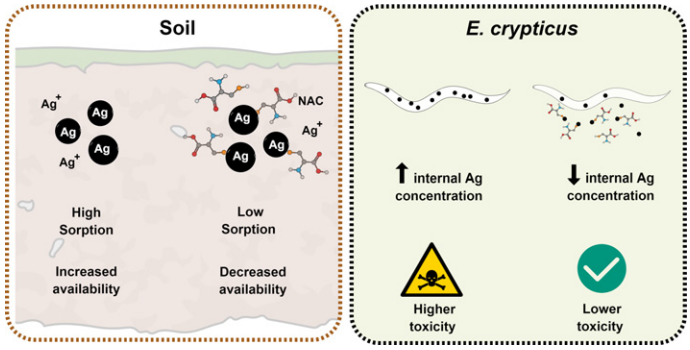
Monique Culturato Padilha Mendonça [a,b,*], Marcelo Bispo de Jesus [a], Cornelis A.M. van Gestel [b]

[a] Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, São Paulo 13083-970, Brazil
[b] Department of Ecological Science, Faculty of Science, Vrije Universiteit, Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, the Netherlands

HIGHLIGHTS

- NAC attenuated the reproduction toxicity induced by AgNPs in a dose-dependent manner.
- The sorption of Ag to soil was strongly reduced in the presence of NAC.
- NAC dose-relatedly decreased the uptake of Ag from the soil by *Enchytraeus crypticus*.
- The formation of soluble Ag-NAC complexes explains the reduced Ag bioavailability.

GRAPHICAL ABSTRACT



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ABSTRACT

We previously demonstrated that *N*-acetylcysteine (NAC) could reduce the toxicity of silver (Ag) materials (nanoparticles (NPs) and Ag nitrate) to the soil invertebrate *Enchytraeus crypticus* (Oligochaeta). It remains however, unclear whether the antitoxic mechanism of NAC was caused by NAC-Ag binding in the soil or inside the organisms. This study aimed at determining the bioavailability of Ag in the soil in a 21-day toxicity test as well as the Ag uptake and elimination kinetics in *E. crypticus* exposed to AgNPs in LUFA 2.2 standard soil amended with low (100 mg/kg dry soil) and high (600 mg/kg dry soil) NAC concentrations. The addition of NAC to the soil alleviated the toxicity of AgNPs by decreasing the internal Ag concentration of *E. crypticus* in a dose-dependent manner. Indeed, NAC reduced the binding of Ag to the soil, which probably was due to the formation of soluble but biologically unavailable Ag-cysteine complexes. The reduced Ag uptake in the enchytraeids was explained from an increased elimination at high NAC levels. These findings reinforce the view that metal complexing-compounds like NAC play a key role in the modulation of AgNP toxicity and bioavailability in terrestrial environments. Further, it may inform on the potential of NAC as a remediation solution for Ag or other metal-contaminated soils.

1. Introduction

The contamination of soils with silver nanoparticles (AgNPs) has become a global problem due to their potential damage to the environment, animal and human health (Tortella et al., 2019). Currently,

* Corresponding author at: Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, São Paulo 13083-970, Brazil.
E-mail address: m115623@dac.unicamp.br (M.C.P. Mendonça).

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nanosilver is used in >443 commercial products (Project on Emerging Nanotechnologies, 2019) of several sectors, including electronic devices, textiles, food packaging, healthcare, cosmetics, and medical devices (Abbasi et al., 2016; Lee and Jun, 2019; Liao et al., 2019). The global production of AgNPs is estimated to be between 500 tons and 1000 tons per year (Calderón-Jiménez et al., 2017; Giese et al., 2018). The more AgNPs are used, the more they can be released into the environment. The potential disposal of industrial and AgNP-based products and waste into the environment could result in an accumulation of AgNPs in the soil, which is known to induce harmful effects on soil invertebrates (Bicho et al., 2016; Schlich et al., 2013; Tourinho et al., 2012), soil microbial communities (Grün et al., 2018; Parada et al., 2019), and plants (Yan and Chen, 2019).

Once released into the environment, AgNPs could undergo physical, chemical and biological transformations that affect their physicochemical properties, thereby changing their toxicity (Levard et al., 2013, 2012; Sharma et al., 2015). One important transformation that affects AgNP toxicity is the chelation of silver ions (Ag^+) with organic and inorganic sulfur-containing compounds, such as cysteine, glutathione, hydrogen sulfide, and S^{2-} (Levard et al., 2012). Cysteine may bind to Ag^+ in biological environments, increasing the dissolution rates of AgNPs and therefore alter their bioavailability to exposed organisms (Behra et al., 2013; Gondikas et al., 2012). *N*-acetylcysteine (NAC) completely prevented the growth inhibition of *Caenorhabditis elegans* caused by AgNPs and $AgNO_3$ (Yang et al., 2012). Cysteine also markedly reduced the toxicity of Ag^+ to *Escherichia coli* (Choi et al., 2018; Xiu et al., 2011).

We recently investigated the use of NAC, a natural thiol-containing antioxidant and an Food and Drug Administration (FDA)-approved mucolytic drug and antidote for acetaminophen overdose (Rushworth and Megson, 2014), for the attenuation of the toxicity of the fully characterized standard reference material AgNM 300K (Klein et al., 2011) and silver nitrate ($AgNO_3$) on the survival, reproduction and avoidance behaviors of the soil invertebrate *Enchytraeus crypticus*. The addition of NAC (600 mg/kg dry soil) to the soil remarkably reduced the toxicity of AgNM 300K and $AgNO_3$, even at high concentrations (Mendonça et al., 2020). However, the underlying mechanism of how NAC mitigates AgNP toxicity to soil invertebrates was not investigated in detail, which greatly limits our understanding of the ecotoxicity of AgNPs in soils. Thus, it is critical to understand the interaction between NAC (thiol groups) and AgNPs and how this alters the biological effects of the latter.

The main goal of this study was to further substantiate our previous findings, by determining the toxicity and bioaccumulation of AgNPs in *Enchytraeus crypticus* in the presence of low (100 mg/kg dry soil) and high (600 mg/kg dry soil) NAC concentrations. We aimed at (1) evaluating the effects of AgNPs on the survival and reproduction of enchytraeids, (2) assessing the sorption of AgNPs to the soil, and (3) determining the uptake and elimination kinetics of Ag in the organisms. This approach may reinforce the view that transformation processes, which include aggregation, dissolution, and surface modifications by metal complexing-compounds, play a key role in the modulation of AgNP toxicity in terrestrial environments.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. The AgNPs used in this study were provided by Applied Nanoparticles SL (Barcelona, Spain) and obtained as a suspension in water containing 5 mM sodium citrate and 1 mg/mL of 55 kDa polyvinylpyrrolidone (PVP). The synthesis has been described previously (Bastús et al., 2014), and the characterization provided by the manufacturer is included in the Supplementary information (Fig. S1). Briefly, transmission electron microscopy images showed that the AgNPs were spherical and mono-dispersed. Dynamic light scattering (DLS) confirmed that the AgNPs were in the nano-range (52.9 ± 4.8 nm), exhibited a Zeta potential (−43.3 ± 5.4) and were well-dispersed (polydispersity index of 0.236 ± 0.009). UV–Vis absorption spectra displayed a maximum absorption peak around 427 nm. Assessments by inductively-coupled plasma-mass spectrometry (ICP-MS) showed an Ag content of 1.8 ± 0.084 mg/mL and approximately $2.2 \cdot 10^{12}$ particles/mL.

2.2. Test organism

This study used the annelid *Enchytraeus crypticus* (Enchytraeidae; Oligochaeta), cultured for several years at the Department of Ecological Science, Vrije Universiteit Amsterdam. Cultures were kept in plastic containers with agar prepared with aqueous soil extract and maintained under controlled conditions (16 °C, 75% relative humidity, and complete darkness). The animals were fed twice a week with a mixture of oatmeal, dried yeast, yolk powder, and fish oil (Castro-Ferreira et al., 2012).

2.3. Test soil

The tests were performed using the standard LUFA 2.2 soil (Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Speyer, Germany), which had 3.5% organic matter, 12% clay, a cation exchange capacity of 9.7 $cmol_c/kg$, a water holding capacity (WHC) of 45.2% and pH (0.01 M $CaCl_2$) of 5.5. The soil was oven-dried at 80 °C for 48 h before use.

Silver NPs were tested at six increasing nominal concentrations: 9.375, 18.75, 37.5, 75, 150, 300 and 600 mg Ag/kg dry soil. For the 300 and 600 mg Ag/kg concentration of AgNPs, spiking had to be performed in steps. Following homogenization, AgNP-spiked soils were kept in a fume hood for 4 h to evaporate the excess amount of water and keep the moisture content of the soil below 50% of the WHC. For reaching appropriate Ag levels, the evaporation procedure was repeated four times for 600 mg Ag/kg, two times for 300 mg Ag/kg, and one time for all the other concentrations. The soil was then stored in glass jars (500 mL) overnight. Subsequently, for each batch of AgNP-spiked soil, NAC was added to give final concentrations of 600 and 100 mg NAC/kg dry soil. These doses were chosen according to our previous study (Mendonça et al., 2020). Controls without AgNPs but with NAC were also included. Tap water was added to give a final moisture content of 45–50% of the WHC, and the soil was left to equilibrate overnight before starting exposures.

2.4. Experimental design

In the first test, we determined the toxicity of AgNPs to the survival and reproduction of *E. crypticus* and assessed the effective concentrations with x% of reduction in reproduction (ECx). In the second test, we determined the toxicokinetics of Ag in the test organisms at a concentration equivalent with the EC_{10} for AgNP toxicity derived from the first test.

All tests were conducted in a climate room at 20 °C, 75% relative humidity and 16-h light/8-h dark photoperiod cycle. The moisture content of the soil was kept constant by replenishing evaporated water with deionized water. Additional food was added once a week.

2.4.1. Toxicity test

The survival and reproduction assay was performed following the OECD guideline for testing of chemicals 220 (OECD, 2015), but using an exposure time of 3 weeks instead of 4 weeks (Castro-Ferreira et al., 2012). Five replicates were used for each treatment. For each replicate, ten adults with well-developed clitellum and of similar size were introduced into a glass jar (100 mL) containing 25 g of moist soil and 2 mg oatmeal as food. The jars were loosely covered with a lid and kept in a climate room. After 21 days, all samples were fixated by adding 10 mL of 96% ethanol. Then, the soil from each test jar was transferred to a plastic container (250 mL) and filled with water (50 mL). Next, animals

were stained using 200 μL 1% Bengal rose solution. The containers were tightly closed, agitated vigorously for 10 s and kept for 6 h at 4 °C. Subsequently, the animals were isolated by sieving through a 180 μm sieve and counted in photo trays using ImageJ software (2006 version, National Institutes of Health, USA).

2.4.2. Toxicokinetics tests

To assess the kinetics of silver uptake, test animals were exposed in soil spiked with AgNPs at 40 mg Ag/kg dry soil, and amended with 0, 100 or 600 mg NAC/kg dry soil. After 10 days of exposure, the remaining animals were transferred to clean LUFA 2.2 soil for assessing the Ag elimination kinetics. For each treatment and sampling time, ten worms were introduced into a 100 mL glass jar filled with 25 g moist test soil, and 2 mg oatmeal. To assess the concentration in the animals at the start of the test (Day 0), *E. crypticus* were sampled from the culture. During the uptake and the elimination phase, sampling took place at days 1, 3, 5, 7 and 10. At each sampling time, three replicate jars were sampled per treatment. The surviving adults were collected and transferred to a 12-well cell culture plate with 1 mL ISO solution (294 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 5.8 mg/L KCl and 64.8 mg/L $NaHCO_3$) during 6 h for gut cleaning. Subsequently, three animals from each replicate were frozen at −20 °C for further Ag analysis.

2.5. Soil and silver analysis

Soil samples (5 ± 1 g), collected at the beginning and end of the tests, were mixed with 0.01 M $CaCl_2$ solution (5:1, w/v) and shaken at 200 rpm for 2 h for pH measurements. After settling of the soil particles (6–8 h), pH was measured in the overlying suspension. Two methods, $CaCl_2$ and porewater extraction, were used to evaluated the effects of NAC on the availability of Ag in the soil. After measuring the pH, the 0.01 M $CaCl_2$ extracts were 0.45-μm filtered and preserved with a few droplets of concentrated HNO_3. Porewater extracts were obtained from each treatment by centrifugation, following the method described previously (Topuz and van Gestel, 2017).

For total Ag measurements, soil samples were dried at 50 °C for 24 h. After drying, approximately 130 mg soil were digested in 2 mL of a 4:1 mixture of HCl (37%) and HNO_3 (65%) in tightly closed Teflon bombs. The bombs were heated for 7 h at 140 °C in a destruction oven. Then, 4 mL of demineralized water was added, and Ag was measured in the solution.

To determine the internal Ag concentrations, animals were freeze dried, individually weighed using an analytical microbalance (Mettler Toledo GmbH UMT2), and transferred into pre-cleaned Pyrex tubes. Then, the worms were digested on a hotplate in 500 μL of a 7:1 mixture of HNO_3 (65%, Mallbaker Ultrex Ultra-Pure) and $HClO_4$ (70%, Mallbaker Ultrex Ultra-Pure). Finally, increasing heating temperatures (85 °C, 130 °C, 160 °C and 180 °C) evaporated all acid. Residues were taken up in 1 mL of 1 M HCl for Ag measurements.

All Ag analyses were conducted by graphite furnace atomic absorption spectrophotometry (GF-AAS) (PinAAcle 900Z, Perkin Elmer) or flame AAS (Perkin Elmer Analyst 100), depending on the concentration level. Two replicates were analyzed per concentration/treatment.

2.6. Data analysis

A logistic dose-response model was used to describe the relationship between juvenile numbers and exposure concentrations (total, 0.01 M $CaCl_2$-extractable, and pore water-extractable Ag concentrations in the soil). From this relationship, we estimated effective concentrations causing 10% and 50% reduction in the number of juveniles per jar (EC_{10} and EC_{50}, respectively) and their corresponding 95% confidence intervals. To compare differences in EC_{50} values, a generalized likelihood-ratio test was applied.

Student *t*-test was applied to compare the differences between control and control-NAC. Since significant differences were found for NAC treatments (low NAC ($p < 0.05$) and high NAC ($p < 0.01$)) compared to the water control, the respective NAC concentrations were used as the control group.

The sorption of silver to the soil was described using a Freundlich isotherm, represented by Eq. (1):

$$C_{sorbed} = K_f \times {C_{ext}}^n \quad (1)$$

where C_{sorbed} is the total Ag concentration (mg/kg dry soil), C_{ext} the Ag concentration in the 0.01 M $CaCl_2$ extract or porewater (mg/L), K_f the Freundlich sorption constant ((L/kg)^n), and n the shape parameter.

A one-compartment model was applied to describe the uptake (Eq. (2)) and elimination (Eq. (3)) kinetics of Ag in the enchytraeids:

$$\text{Cworm} = C0 + \frac{k1}{k2} \times Cexp \times \left(1 - e^{-k2t}\right) \quad (2)$$

$$\text{Cworm} = C0 + \frac{k1}{k2} \times Cexp \times \left(e^{-k2(t-tx)} - e^{-k2t}\right) \quad (3)$$

where *C*worm is the internal Ag concentration in the enchytraeids at time t (mg/kg dry body weight), C_0 is the initial (background) Ag concentration in the enchytraeids at $t = 0$ (mg/kg dry body weight), k_1 is the uptake rate constant (kg soil/kg worm/day), k_2 is the elimination rate constant (day^{-1}), *C*exp is the Ag exposure concentration during the uptake phase (mg/kg dry soil), t is the exposure time (days), and tx is the day on which animals were transferred to clean LUFA 2.2 soil (day 10). Both Eqs. (2) and (3) were fitted together to obtain single values for the uptake and for the elimination rate constants. The bioaccumulation factor (BAF) for the accumulation of the Ag in *E. crypticus* was estimated as $\text{BAF} = \frac{k1}{k2}$.

Microsoft Excel 2010 was used to fit the one-compartment model, and IBM SPSS Statistics 21 to estimate the standard errors and other statistical parameters. Inter-group statistical comparisons were made by One-way analysis of variance (ANOVA) with Dunnett's post hoc test.

3. Results

3.1. Ag availability in the test soils

Measured total soil concentrations in the toxicity test were within 10% of nominal Ag concentrations (Table S1), indicating the validity of the dispersion and dosing protocols. All data analysis was conducted using measured Ag concentrations. Soil spiked with AgNPs had pH_{CaCl2} ranging between 6.32 and 6.50, and pH was not much affected by NAC addition (6.00–6.53).

Table 1 lists the sorption parameters of the AgNPs in LUFA 2.2 soil with and without NAC. The Freundlich adsorption constant (K_f) for the relation between total soil and $CaCl_2$-extractable Ag concentrations without NAC (1533 (L/kg)^n) was one order of magnitude higher than for low (192 (L/kg)^n) and high (138 (L/kg)^n) NAC. The n values were inversely proportional to the NAC concentration, confirming a strong reduction of Ag sorption in the presence of high levels of NAC. After 21 days, we observed an increase in the K_f values of NAC-spiked soils compared to Day 0. All adsorption isotherms were nonlinear, with n

Table 1
Freundlich isotherm parameters (±SE) for the sorption of Ag from AgNPs to LUFA 2.2 soil in the absence and presence of low and high *N*-acetylcysteine (NAC) concentrations, based on 0.01 $CaCl_2$ extracts.

Groups	Day 0			Day 21		
	$K_f \pm$ SE	$n \pm$ SE	R^2	$Kf \pm$ SE	$n \pm$ SE	R^2
No NAC	1533 ± 2.7	0.97 ± 0.31	0.45	1191 ± 1.6	0.75 ± 0.12	0.76
Low NAC	192 ± 1.2	0.59 ± 0.07	0.86	506 ± 1.2	0.49 ± 0.04	0.95
High NAC	138 ± 1.2	0.42 ± 0.07	0.81	513 ± 1.1	0.26 ± 0.02	0.96

values <1. Silver concentrations in pore water extracts and resulting Freundlich sorption parameters demonstrated the same trend as in $CaCl_2$ extracts (Table S2).

3.2. Survival and reproduction

The test satisfied the validity criteria established in the OECD 220 guideline (OECD, 2015), i.e., adult mortality ≤ 20%, juvenile production ≥ 25 per replicate, and a corresponding coefficient of variation (CV) lower than 50%. In control replicates, the survival of *E. crypticus* was above 98%, >942 juveniles per jar were produced, and the CV was 15.2%. The survival rate of AgNP-exposed animals was not significantly different from those of the control, even at the highest concentration of 600 mg Ag/kg; thus, no LC_{50} (lethal concentration, 50%) could be calculated. Neither low (82% ± 3.74) nor high (90% ± 3.16) NAC alone or combined with AgNPs affected enchytraeid survival (Table S3).

The fitted concentration-response curves for the effects of AgNPs without and with low or high NAC concentrations on the reproduction of *E. crypticus* after 21 days of exposure are shown in Fig. 1. The number of juveniles decreased with increasing AgNP concentrations in the soil. The addition of low and high NAC concentrations in the soil mitigated AgNP toxicity to the worms in a dose-dependent manner (Fig. 1, Table S4). Corresponding EC_{50} and EC_{10} values are presented in Table 2.

High NAC exposure increased the EC_{50} for the toxicity of AgNPs by ~6 fold compared to no NAC ($\chi^2_{df=1} = 13.30$, $p < 0.001$) and ~5 fold compared to low NAC ($\chi^2_{df=1} = 12.75$, $p < 0.001$). In contrast, low NAC exposure produced only a mild increase in EC_{50} compared to AgNPs without NAC (176 vs. 157 mg Ag/kg dry soil, respectively). Relative toxicity was also evaluated by comparing the slope of the dose-response curves. For AgNPs without and with low NAC, the slopes were not significantly different (overlapping 95% confidence limits). The low slope of the concentration-response curve for the high NAC group however, was flatter and, consequently, EC_{10} values were similar between the different NAC treatments.

Table 2
Estimated effect concentrations (EC) for the effect of AgNPs on the reproduction of *Enchytraeus crypticus* at different doses of *N*-acetylcysteine (NAC) in LUFA 2.2 soil, estimated using a logistic dose-response model; 95% confidence intervals are reported in between brackets.

	EC_{50} (mg Ag/kg dry soil)	EC_{10} (mg Ag/kg dry soil)	Slope
No NAC	157 (123–190)	40 (19–61)	1.61 (1.13–2.09)
Low NAC	176 (136–217)	25 (10–39)	1.12 (0.85–1.39)
High NAC	937 (322–1552)	12 (−12–36)	0.51 (0.27–0.75)

3.3. Toxicokinetics

No worms died throughout the uptake-elimination kinetics tests. All worms gained weight during the 20-day test period; exhibiting at day 1 and day 20, respectively, average masses of 0.74 ± 0.04 and 1.09 ± 0.18 mg d.w. in soils without NAC, 0.80 ± 0.15 and 0.97 ± 0.08 mg d.w. in soils with low NAC, and 0.88 ± 0.10 and 1.02 ± 0.10 mg d.w. in soils with high NAC concentrations. Mass change during the study was not significant to justify modification of the kinetics equations by including a growth rate constant (to cope with the effect of growth dilution on body Ag concentrations). Total Ag content in the soil (mg/kg, d.w.) was 39.5 for no NAC, 36.4 for low NAC and 36.7 for high NAC-spiked soils. The initial body Ag concentrations in the enchytraeids were 0.05–0.07 mg Ag/kg d.w. The internal Ag concentrations of the worms kept in the control soil were 0.03–0.13 mg Ag/kg d.w. during the uptake and elimination phases. As displayed in Fig. 2, the average internal Ag concentrations of the enchytraeids exposed to AgNPs without NAC (blue line) soil reached up to 20.5 ± 3.89 mg Ag/kg d.w. at the end of the uptake phase (average of days 7 and 10). When transferred to clean soil, body Ag concentrations rapidly decreased to 2.22 mg Ag/kg

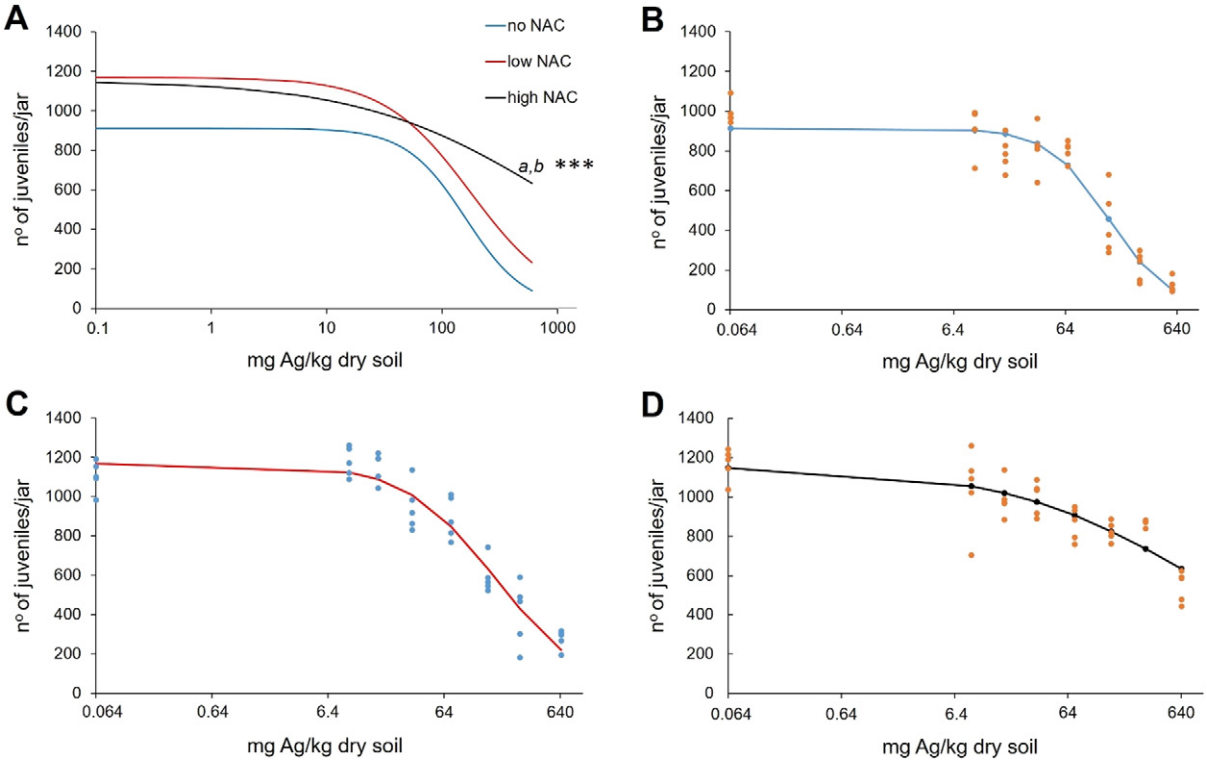

Fig. 1. (A) Fitted concentration-response relationships for the effect of NAC on the reproduction of *Enchytraeus crypticus* after 21 days of exposure in AgNP-spiked LUFA 2.2 soil. Frequency distributions of the data sets from the animals treated (B) without NAC, (C) with 100 mg NAC/kg dry soil, and (D) with 600 mg NAC/kg dry soil used to obtain the dose-response relationships shown in (A). The number of juveniles per jar is plotted on the *y*-axis, measured log-transformed Ag concentrations are plotted on the *x*-axis. *a*: EC_{50} significantly different compared to no NAC ($p < 0.001$); *b*: EC_{50} significantly different compared to low NAC ($p < 0.001$), according to a generalized likelihood ratio test.

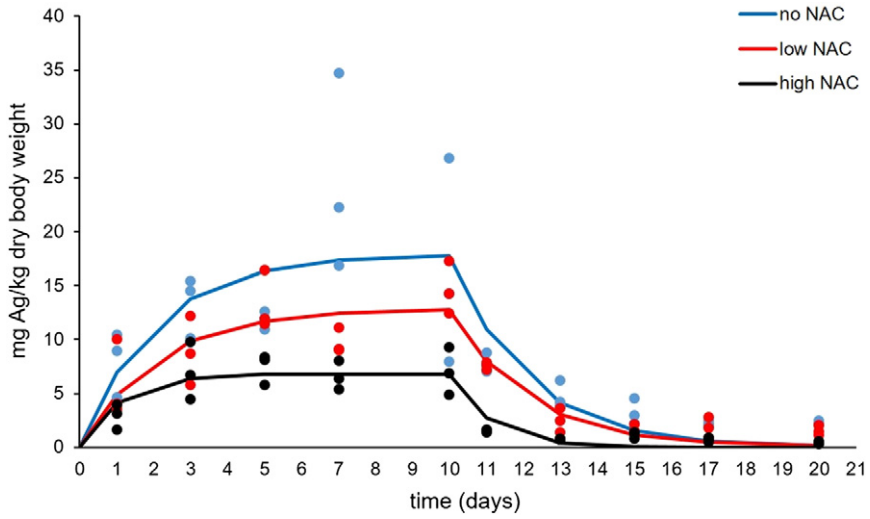

Fig. 2. Effect of *N*-acetylcysteine (NAC) on the uptake and elimination kinetics of Ag in *Enchytraeus crypticus* exposed for 10 days to AgNPs (at nominal 40 mg Ag/kg dry soil) in LUFA 2.2 soil, followed by 10 days elimination phase in clean LUFA 2.2 soil. Lines show the fit of a one-compartment model to the data (Eqs. (1) and (2)), without NAC (blue), low NAC (red), and high NAC (black).

dry d.w. The body Ag concentrations reached (average of days 7 and 10) were dose-related and significantly lower when NAC was added to the soil, being 12.21 ± 1.30 Ag/kg d.w. for low NAC ($p < 0.05$) (red line) and 6.8 ± 0.67 Ag/kg d.w. for high NAC ($p < 0.01$) (black line) compared to enchytraeids exposed to AgNPs without NAC in the soil. For AgNPs without NAC (blue line) and with low NAC (red line), Ag uptake by the enchytraeids did not reach equilibrium after 10 days exposure, however, at the high NAC concentration (black line) steady-state was achieved within 3 days of exposure. Table 3 shows the corresponding uptake and elimination rate constants.

The internal Ag concentrations were higher in the enchytraeids exposed to AgNPs without NAC, but the k_1 values did not differ much from the low and high NAC treatments. The elimination rate constants suggested fast Ag elimination. The k_2 was highest for the high NAC treatment, but not significantly different from the treatment without NAC given the overlap in confidence intervals. Similar k_2 values were found for exposures without and with low NAC. Bioaccumulation factor (BAF) values clearly decreased with increasing NAC concentrations and were below 1 for all treatments.

4. Discussion

This study aimed at using a kinetics approach to determine the availability and bioaccumulation of AgNPs to *Enchytraeus crypticus* in the absence and presence of two concentrations of NAC, with a focus on understanding the dose-dependent role of NAC on AgNP toxicity. In this study, survival was a less sensitive endpoint than reproduction, which has also been reported previously (Gomes et al., 2013; Heckmann et al., 2011; Schlich et al., 2013; Topuz and van Gestel, 2017). A dose-dependent decrease in the number of juveniles was observed after exposure to AgNPs for 21 days. Similar to our observations, various studies have reported the toxicity of AgNPs to the reproduction of a diverse range of soil invertebrates including the annelids *Enchytraeus albidus* (Gomes et al., 2013) and *E. crypticus* (Topuz and van Gestel, 2017), the earthworms *Lumbricus rubellus* (Makama et al., 2016) and *Eisenia andrei* (Jesmer et al., 2017), and the springtail *Folsomia candida* (McKee et al., 2017; Waalewijn-Kool et al., 2014).

Several management practices are being applied to minimize the risk of contamination of soils with toxic metals by decreasing their availability and/or bioaccumulation in living organisms. These treatments include the use of chelating compounds, natural sorbents (e.g. farmyard manure, saw dust and rice husk) and nanomaterials (Singh and Prasad, 2015). In this study, we used NAC, as a potential thiol chelation agent for Ag-contaminated soils. In addition to its role as a Ag^+ chelator (Gondikas et al., 2012), NAC can also serve as an antioxidant able to prevent the generation of reactive oxygen species induced by AgNPs, which is one of the mechanisms involved in AgNP toxicity to soil invertebrates (Gomes et al., 2015; Hayashi et al., 2013; Ribeiro et al., 2015a, 2015b). The addition of NAC to soil alleviated AgNP toxicity to *E. crypticus* in a dose-dependent manner. Reproduction EC_{50} increased from 157 mg Ag/kg dry soil to 176 mg/kg in low-NAC and 937 mg/kg in high-NAC treated soils (Table 2). Although a low dose of NAC already reduced the toxicity of AgNPs, the effect of NAC on reproduction was only significant at the higher dose. Several studies described the benefits of NAC supplementation in mammals, e.g. to improve the male reproductive potential (Calogero et al., 2017; Jannatifar et al., 2019). As a powerful metal chelator (Rossignol, 2018), the coadministration of NAC ameliorates the harmful effects of the arsenic trioxide on the reproductive tract of male mice (Da Silva et al., 2016). In aquatic invertebrates, NAC was able to recover the spermiotoxic effects of copper oxide NPs (Gallo et al., 2018). Ma et al. (2014) suggested that NAC may mitigate the toxicity of metallic NPs to invertebrates through multiple mechanisms including chelation of free ions and thus reducing their bioavailability and toxicity and/or by reducing oxidative stress through its antioxidant properties (Ma et al., 2014).

In our study, one reason for the reduced toxicity in the presence of NAC might be a stronger retention of the AgNPs by the soil. To test for that, binding strength of the Ag to the soil was determined by assessing 0.01 M $CaCl_2$ extractable and porewater Ag concentrations. In both cases, however, sorption seemed to be reduced rather than enhanced by the addition of NAC (Tables 1 and S2). Considering the high water-solubility of NAC, this reduction may apparently be attributed to the

Table 3
Kinetics parameters for the uptake and elimination of Ag in *Enchytraeus crypticus* following exposure to AgNPs (at nominal 40 mg Ag/kg dry soil) in LUFA 2.2 soil without and with low or high concentrations of *N*-acetylcysteine (NAC). k_1 is the uptake rate constant, k_2 the elimination rate constant, and BAF is the bioaccumulation factor. A one-compartment model was used to estimate kinetics parameters, using Eq. (2) for uptake and Eq. (3) for elimination phase data. 95% confidence intervals are reported in brackets.

	No NAC	95% CI	Low NAC	95% CI	High NAC	95% CI
k1 (kg soil/kg worm d.w./day)	0.22	(0.13–0.32)	0.17	(0.12–0.22)	0.17	(0.11–0.24)
k2 (day^{-1})	0.49	(0.27–0.73)	0.48	(0.34–0.62)	0.94	(0.56–1.32)
BAF	0.45		0.35		0.18	

formation of water-soluble complexes, hence making Ag not available in the solution for affecting the enchytraeids. It is well known that chelating agents may desorb metals from the soil solid phase by forming water-soluble complexes (Singh and Prasad, 2015). Furthermore, the interaction of NPs within the natural organic matter (NOM) also plays an important role not only in controlling the environmental transformation, transport, and mobility of the NPs in the environment, but also in influencing their toxicity to organisms that might potentially be affected (Baalousha et al., 2018; Grillo et al., 2015; Yang et al., 2014). Several studies indicated that the addition of NOM to nanomaterials resulted in lower toxicity to the majority of organisms tested (nematodes, bacteria, algae, microcrustaceans, and fish) (Collin et al., 2016; Grillo et al., 2015).

Cysteine represents a class of naturally ubiquitous organic ligands (-SH, thiol group), which is commonly present in NOM (Pokhrel et al., 2013; Rao et al., 2014) and was used as a low molecular weight model for NOM (Gondikas et al., 2012; Yang et al., 2014). Although we cannot fully explain the sorption reduction enhanced by the addition of NAC from the data obtained from our study, three hypotheses have been proposed for the reduced toxicity in the presence of NAC: (i) the NOM (i.e., NAC) may coat the AgNPs and therefore limit their interaction with or uptake by the test organisms, (ii) the NOM coating may limit the release of toxic metal ions from the AgNPs, or (iii) the NOM may act as a scavenger and bind the released Ag^+. At high ionic strengths (300 mM), PVP-AgNP aggregation was enhanced by L-cysteine (a simple model of NOM) due to its binding to the AgNPs and replacement of the steric stabilizing agent PVP, thus weakening the mobility of PVP-AgNP complexes in porous media (Yang et al., 2014). The impact of cysteine on the release of Ag^+ has been described by Priester et al. (2014). Capping the AgNPs with L-cysteine provides higher stability and slower Ag^+ release compared to $AgNO_3$, resulting in different toxicities to bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) that was mainly attributed to the release of Ag^+ and not intact nano-Ag (Priester et al., 2014). Compounds with free thiols, such as glutathione and cysteine, inhibit ion release presumably by surface binding and oxygen exclusion from active sites (Liu et al., 2010).

After measuring Ag availability in the soil, the next important step was to assess its uptake rate in the test organisms. The body Ag concentrations confirmed that NAC induced a dose-dependent decrease in the Ag bioaccumulation. In the uptake phase, only at the high NAC concentration a steady-state body concentration was reached within the 10-day exposure period, while body Ag concentrations were still increasing with time for the low-NAC and no-NAC exposures (Fig. 2). Internal Ag concentrations (from day 3) in *E. crypticus* were around 7 mg/kg d.w. at high NAC, while they ranged between 5.86 (day 1) and 14.7 mg/kg d.w. after 10 days for low NAC. These findings suggest rapid attachment of NAC to the surface of the AgNPs; once the NPs were fully covered, increasing NAC concentration did not change the uptake dynamics over time. A recent study reported that cysteine concentrations ranging from 5 to 5000 μM resulted in different effects on total Ag uptake by a fungus exposed to AgNPs or Ag^+; a decrease in total Ag uptake was observed at 5–50 μM cysteine in the fungus exposed to 10 μM AgNPs and 1 μM Ag^+, especially at a Cys:Ag molar ratio of 5 (Huang et al., 2018). Assuming that Ag uptake followed first-order kinetics and considering that the worms behaved as one compartment, we calculated uptake (k_1) and elimination (k_2) rate constants and the BAF for Ag (Table 3). Although NAC reduced the internal Ag concentration (Fig. 2), the uptake rate constants were quite similar for all groups (Table 3). The NAC-Ag interaction apparently was more complex than previously hypothesized. Why are uptake patterns of AgNPs with and without NAC similar? Obviously, the NAC protective mechanism may depend on the concentration of Ag inside the organism, rather than the rate at which Ag is entering the organism. For metal NPs, size, shape, and surface properties play an important role in cellular uptake kinetics. Generally, smaller NPs are internalized more efficiently than larger ones with similar surface characteristics (Panzarini et al., 2018). Luoma et al. (2016) demonstrated that a single Ag-cysteine complex is small but large cysteine-AgNP aggregates can also be created via hydrogen bond formation between amino acid molecules located on neighboring Ag particles. Our findings suggest that NAC chelated nanoparticulate Ag in the surrounding medium forming NAC-Ag complexes not accessible for uptake. The remaining uptake was of NAC-Ag complexes that promoted AgNP aggregation/agglomeration to a certain extent, resulting in similar uptake rate constants since Ag aggregates/agglomerates enter at a slow rate. On the other hand, the lower elimination rate constant of Ag without NAC (0.49 day^{-1}) and with low NAC (0.48 day^{-1}) compared to high NAC (0.94 day^{-1}) indicates that higher levels of NAC improved the depuration of Ag from the enchytraeid body, leading to lower BAF values. In many species (e.g. annelids, mollusks, crustaceans, fish), Ag is known to be associated with metallothioneins (Amiard et al., 2006) and form metal-rich granules that store Ag in such a way that it cannot be eliminated anymore, or be eliminated only at a rather slow rate. Therefore, depuration of Ag is less efficient than its accumulation in the organism and Ag may be accumulated till it reaches toxic levels. Such binding to metallothioneins probably is only relevant for ionic Ag forms and less expected for Ag complexes or particles (Ribeiro et al., 2015a, 2015b). Given that this is the first study, as far as we know, evaluating the influence of NAC on the uptake and elimination kinetics of AgNPs by enchytraeids, it is difficult to discuss our findings in the context of similar studies.

5. Conclusions

This study demonstrated that the toxicity to enchytraeid reproduction of AgNPs may be directly linked with high Ag availability in the soil therefore resulting in increased Ag bioaccumulation. Although the addition of NAC reduced the sorption of Ag to the soil, it alleviated the toxicity of AgNPs by decreasing the uptake of Ag in *E. crypticus*, however, without changing the uptake rate. This most likely is due to the formation of soluble Ag-NAC complexes not available for uptake by the enchytraeids. In this way NAC protects the enchytraeids from toxicity of the AgNPs by reducing the concentration of Ag inside the organisms, rather than the rate at which Ag is entering the organism. These effects of NAC occurred in a dose-dependent manner. Mechanisms that underlie this complex behavior require further study. Overall, we conclude that NAC could be an effective alternative for early remediation and recovery of terrestrial organisms exposed to metal-contaminated soils.

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.

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Appendix A. Supplementary data

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