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Environmental Toxicology

The Increase in Temperature Overwhelms Silver Nanoparticle Effects on the Aquatic Invertebrate *Limnephilus* sp.

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Abstract: The effects of silver nanoparticles (AgNPs) have been largely explored, but there is still a lack of knowledge on their effects under the predicted changes in temperature as a consequence of climate change. The aim of the present study was to determine how leaf consumption by invertebrate shredders is affected by dietary exposure to AgNPs and $AgNO_3$ and whether changes in temperature alter such effects. Also, responses of antioxidant enzymes were examined. In microcosms, the invertebrate shredder *Limnephilus* sp. was allowed to feed on alder leaves treated with AgNPs (5, 10, and 25 mg L^{-1}) and $AgNO_3$ (1 mg L^{-1}) at 10, 16, and 23 °C (6 replicates). After 5 d, the animals were transferred to clean water and allowed to feed on untreated leaves. The higher leaf consumption by the shredder was related to temperature increase and to the contamination of leaves with AgNPs and $AgNO_3$. Results from enzymatic activities demonstrated that AgNP contamination via food induce oxidative and neuronal stress in the shredder: the activities of catalase and superoxide dismutase were positively correlated with total Ag accumulated in the animal body. Moreover, glutathione *S*-transferase activity was strongly associated with higher temperature (23 °C). Overall results indicated that the effects of toxicants on consumption rates and enzymatic activities are modulated by temperature and suggested that increases in temperature changes the AgNP effects on invertebrate shredder performance. *Environ Toxicol Chem* 2020;00:1–9. © 2020 SETAC

Keywords: Silver nanoparticles; Temperature; Dietary exposure; Leaf litter consumption; Aquatic invertebrate shredders

INTRODUCTION

Climate projections show a temperature increase over Europe from 1 to 4.5 °C, under a moderate scenario, and from 2.5 to 5.5 °C, under the worst-case scenario, by the end of the century (European Environment Agency 2017). Moreover, according to a report from the Intergovernmental Panel on Climate Change (2014), there is also high confidence in changes in temperature extremes. These projections are expected to threaten biodiversity, with consequences to ecosystem functions and services (Vörösmarty et al. 2010). Forest streams, where water temperature is typically low throughout the year, are expected to be more vulnerable to the increase in temperature (Dudgeon et al. 2006), with particular impacts on the survival of cold-water species, including invertebrates (Haidekker and Hering 2007).

Invertebrates play an important role in streams, supporting several functions such as the processing of organic matter, nutrient cycling, and secondary productivity (Wallace et al. 1996). In low-order forest streams, invertebrate shredders actively contribute to the fragmentation of plant material by transforming coarse particulate organic matter into fine particulate organic matter (Webster and Benfield 1986), which can be used by filter-feeders and collector-gatherers (Graça 2001). Many species of freshwater invertebrates are highly sensitive to changes in temperature (Haidekker and Hering 2007; Jonsson et al. 2015) and consequently more prone to extinction attributable to climate change (Conti et al. 2014).

An increase in water temperature can affect invertebrates by promoting higher initial growth rates, shorter developmental time, and smaller size at maturity (Atkinson 1995), as well as changes in the feeding behavior of invertebrate shredders (Ferreira et al. 2010; Silva et al. 2018). These effects are predicted to be stronger for invertebrates inhabiting cold waters when compared to those inhabiting warmer waters (Braune et al. 2008) because biological activities are more temperature-limited in cold-water environments (Brown et al. 2004). Invertebrates interact with other groups of organisms, from primary producers to top predators, so alterations in their activities may have impacts on ecosystem functioning under climate change (Traill et al. 2010).

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In freshwaters, the effects of changes in temperature can alter the impacts of other stressors, such as eutrophication and metal pollution (Dudgeon et al. 2006; Batista et al. 2017a). Silver nanoparticles (AgNPs) are widely used in daily consumer products, such as personal care products, water disinfectants, air filters, and textile fabrics, mainly because of the antibacterial properties of both nano and ionic forms of Ag. The rapidly expanding production and use of AgNPs explain their presence in aquatic environments, where AgNPs and ionic Ag released from NPs can have toxic effects on aquatic biota (review: Fabrega et al. 2011; green algae: Oukarroum et al. 2012; invertebrate shredders: Batista et al. 2017b).

The predicted environmental concentrations for AgNPs in surface waters in 2017 were within the range of 0.03 to 2.79 ng L^{-1} (Giese et al. 2018), but higher concentrations can be attained during accidental spills or in wastewaters (Gottschalk et al. 2013). For example, AgNPs have been released from commercially available socks into wastewater treatments in concentrations >1.2 mg L^{-1} Ag (Benn and Westerhoff 2008). Moreover, NPs can be dispersed by wind and rain events (Domercq et al. 2018), potentially putting at risk even low-order forest streams. Once in freshwaters, NPs can be ingested by invertebrate shredders via contaminated water or food, with negative impacts on their fitness (Pradhan et al. 2015; Batista et al. 2017b).

The impacts of AgNPs on invertebrate detritivores have been mainly assessed by acute toxicity (Zhao and Wang 2012; Andreï et al. 2016). The invertebrate feeding behavior has been indicated as a useful tool for assessing NP toxicity, but this parameter is less recognized in ecotoxicological studies (but see Pradhan et al. 2015; Ray 2016; Batista et al. 2017b; Lüderwald et al. 2019). Also, exposure to toxicants triggers physiological and biochemical responses to help organisms to face stressors. In organisms, AgNPs can induce oxidative stress by increasing the production of reactive oxygen species (ROS; e.g., Walters et al. 2016; Barros et al. 2019), which can damage macromolecules in cells, including proteins, lipids, and DNA (Halliwell and Gutteridge 1999). Enzymes with antioxidant activities (e.g., catalase [CAT] and superoxide dismutase [SOD]) and with neuronal activities (acetylcholinesterase [AChE]) triggered by environmental stressors are considered early warning biomarkers of stress (Ray 2016).

The specific properties of AgNPs (e.g., size, aggregation state) influence their toxicity, which may also vary with environmental factors, including temperature. However, the effects of AgNPs and water temperature in aquatic ecosystems have been mostly examined independently, and little information on their combined effects is available (but see for algae: Oukarroum et al. 2012; microbial decomposers: Batista et al. 2017a). In a previous study, we showed that AgNPs or Ag^+ inhibit the activity and diversity of aquatic microbial decomposers of plant litter, mainly when temperature increases from 16 to 23 °C (Batista et al. 2017a).

In the present study, we aimed to determine how plant litter consumption by invertebrate shredders is affected by AgNPs and whether changes in temperature alter these effects. The toxicity of AgNPs to invertebrate shredders was compared to that of Ag^+ to disentangle the contribution of the nano and ionic forms to overall toxicity. To clarify the mechanisms underlying possible toxic effects of AgNPs or Ag^+ on shredders, we examined the responses of antioxidant and neuronal stress enzymes under changes in temperature. We expected that 1) the rise in temperature would stimulate leaf consumption by the shredders; 2) the ingestion of AgNPs or Ag^+ via treated leaves would induce oxidative and neuronal stress, especially at higher concentrations; 3) changes in temperature would alter AgNP effects on the feeding activity of aquatic invertebrates; and 4) invertebrate feeding and enzymatic activities would recover after transfer of animals to clean water and feeding them on leaves untreated with AgNPs or Ag^+.

MATERIAL AND METHODS

Collection of invertebrates and acclimation to the laboratory

Experiments were performed with early-stage larvae common in Iberian streams: the shredder *Limnephilus* sp. (Limnephilidae, Trichoptera; Batista et al. 2012). Animals were collected in the Cávado River, Portugal (41°48′N, 7°51′W), and acclimated for 2 wk in the laboratory under sterilized (121 °C, 20 min) and aerated stream water at 16 °C (±2 °C) and 140 rpm, with a supply of *Alnus glutinosa* (L.) Gaertn. (alder) leaves.

Total inorganic carbon in the stream water was 3.6 ± 0.1 mg L^{-1}, and dissolved organic carbon was below the detection limit (0.5 ppm). Also, the stream water contained silica, 9.6 ± 2 mg L^{-1}; Na^+, 4.1 ± 0.4 mg L^{-1}; K^+, 0.6 ± 0.1 mg L^{-1}; Ca^{2+}, 1.3 ± 0.3 mg L^{-1}; Cl^-, 4.2 ± 0.4 mg L^{-1}; HCO_3^-, 8.0 ± 0.8 mg L^{-1}; and SO_4^-, 1.0 ± 0.2 mg L^{-1} (ionic strength: 2.2 ± 0.1 mmol L^{-1}; Pradhan et al. 2015).

AgNP characterization and metal analysis

The AgNPs were citrate-coated (20 nm, NanoSys) and the ionic form was $AgNO_3$ (>99%; Sigma-Aldrich). The AgNP hydrodynamic diameters and surface charges (zeta-potential) were measured in all suspensions by dynamic light scattering using a Zetasizer (Nano ZS; Malvern Instruments), as in Batista et al. (2017a).

The total Ag concentration (isotope ^{109}Ag) in the water and in leaves was determined, after acid digestion, by inductively coupled plasma mass spectrometry (Thermo Scientific XSERIES 2; detection limit <0.1 µg L^{-1}) at the Scientific and Technological Research Assistance Centre (University of Vigo, Spain). The total Ag concentration was determined by the same method in the animal tissue (pool of the 2 animals used per replicate).

Acute lethality tests

The sensitivity of the invertebrate shredders to aqueous AgNPs or $AgNO_3$ was evaluated by acute lethality tests; this allowed us to select the exposure concentrations in further assays. Invertebrates (3 animals per replicate, 3 replicates per concentration) were starved for 24 h before being placed in

250-mL Erlenmeyer flasks with 130 mL of water supplemented or not with AgNPs (8 levels, 0.05–75 mg L^{-1}) or $AgNO_3$ (8 levels, 0.005–7.5 mg L^{-1}). Animals were kept for 96 h at 16 °C, under a 12:12-h light:dark photoperiod, with no food provided. Every 24 h, dead animals were determined by the absence of movement after mechanical stimulation.

Exposure feeding experiment

Alder leaf disks were immersed in a clean stream for 10 d for microbial colonization. Leaf disks were then exposed or not to AgNPs and $AgNO_3$ at 10, 16, and 23 °C for 21 d, before being offered to the animals. Temperatures were selected, taking into account that 16 °C is a temperature commonly found in streams of northwest Portugal in early autumn and 10 and 23 °C to simulate possible temperature extremes (Batista et al. 2017a).

Microbially colonized leaves (sets of 16 leaf disks, 12 mm diameter) treated or not with AgNPs (5 mg L^{-1}, >10% lethal concentration [LC10]; 10 mg L^{-1}, LC20–LC30; and 25 mg L^{-1}, LC60–LC80) or $AgNO_3$ (1 mg L^{-1}, LC10–LC20) were placed in 250-mL Erlenmeyer flasks suplemented with 150 mL of stream water (without AgNPs or $AgNO_3$) and containing 2 animals ($14.5 \times 1.8 \pm 1$ mm length), starved for 24 h. Microcosms were incubated for 5 d, under aeration and a 12:12-h light:dark photoperiod, at 10, 16, or 23 °C. Six replicates were used per treatment, 30 microcosms per temperature (total of 90 microcosms). Animal survivorship was registered every 12 h.

At the end of the experiment, 3 replicates were used to quantify leaf consumption and the enzyme activity in the shredders. Leaf disks were lyophilized to a constant weight and weighed before and after the feeding experiment. Animals were separated from their cases, frozen immediately in liquid nitrogen to prevent cellular metabolism, and kept at –80 °C until the enzymatic assays. The same animal samples were used to determine Ag concentration in the animal bodies.

Postexposure feeding experiment

After the feeding experiment, animals remaining from a set of 3 replicates per treatment were recovered from nano or ionic Ag stress; the animals were transferred to microcosms with clean stream water and allowed to feed on untreated microbially colonized leaves (10–12 disks per microcosms). Animals were kept for 3 d under aeration and photoperiod conditions described in the section *Exposure feeding experiment*. At the end of the experiment, leaf disks were lyophilized to constant weight, and animals were kept at –80 °C for further assays. Leaf disks were weighed before and after the postexposure feeding experiment. The same animal samples were used to determine Ag concentration in the animal bodies.

Activity of antioxidant and neuronal enzymes

The activities of the antioxidant (glutathione *S*-transferase [GST], CAT, SOD) and neuronal (AChE) enzymes were determined in the animal body and head, respectively, as described in Pradhan et al. (2016). Briefly, the heads and the bodies were separated, after freezing the animals, and then homogenized (Ultraturrax IKA) in 0.1 M K-phosphate buffer (1:8), pH 7.4, at 4 °C. The homogenates were centrifuged (10 000 *g* for 20 min, at 4 °C) to separate the postmitochondrial supernatant (PMS). The PMS from body or head tissues was divided into aliquots and stored at –80 °C for protein quantification and evaluation of the activities of stress responsive enzymes.

The measurement of CAT activity was based on the dismutation of hydrogen peroxide and detected at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$), according to a modified method of Claiborne (1985). The activity of GST was determined based on the formation of 1-glutathione-2,4-dinitrobenzene and detected at 340 nm ($\varepsilon = 9.6\ mM\ cm^{-1}$), as described by Habig et al. (1974). The quantification of SOD activity was based on its ability to inhibit superoxide radical–dependent reactions as described in the Ransod Kit (Randox Laboratories). The activity of AChE was determined based on the production of thiocholine hydrolyzed from acetylthiocholine and detected at 414 nm ($\varepsilon = 13.6\ mM\ cm^{-1}$), according to the Ellman method (Ellman et al. 1961).

Enzymatic activities were expressed in nanomoles per minute per milligram of protein, except SOD which was expressed as SOD units per milligram of protein. Protein concentration in PMS was quantified according to the method of Bradford (1976), using bovine serum albumin as standard. The protein concentration was expressed per wet mass of animal.

Data analyses

Mortality of invertebrate shredders was recorded, and the concentration inducing death (LC) after 96 h of exposure with the respective 95% CI was calculated using PriProbit 1.63 (Sakuma 1998). Probit analysis is based on linear regression analysis of probit-transformed proportion survival data and the log10 of the concentration.

The effects of temperature and nano or ionic Ag concentrations on leaf consumption, Ag accumulation, and o enzymatic activities were tested by 2-way analysis of variances (ANOVAs), followed by Bonferroni post hoc tests (Zar 2010). Raw data were used because they were normally distributed and homoscedastic. GraphPad Prism 5 (GraphPad Software) was used for all statistical tests described.

Consumption rates (grams of leaf dry mass per gram of animal wet mass per day) were calculated as consumption rate = Le/(WMf × t), where Le is the leaf dry mass consumed during the elapsed time (t) and WMf is the final wet mass of animals (Batista et al. 2012).

Principal component analysis (PCA) was used to establish the relationships between AgNP concentration, $AgNO_3$ concentration, temperature, total Ag accumulated in the animal body, and enzymatic activities after exposure and postexposure feeding experiments. A correlation matrix was used to standardize the data and calculate the principal components (PCs). Significant effects were assessed by permutational

multivariate ANOVA. The PCA was done in PAST 3.11 for Windows (Hammer et al. 2001).

RESULTS

AgNP charaterization and metal analysis

The surface charges and characterization of hydrodynamic diameters of AgNPs in water can be found in Batista et al. (2017a). Initially, the average size of AgNPs was 26 ± 3 nm, and it had a tendency to increase at higher temperatures (23 °C). The surface charge (zeta-potential) decreased with increasing AgNP concentrations.

After 21 d, total Ag concentrations in the water of microcosms with no added Ag exposed to all temperatures were below the detection limit (>5 μg L^{-1}). The total Ag concentration in the water with AgNPs was between 60 and 100% of the concentration added as NPs (Batista et al. 2017a). After 21 d, the total Ag concentration in control leaves exposed to all temperatures was below the detection limit (<5 μg g^{-1}). Total Ag concentration in the leaves exposed to AgNPs increased with the increase in AgNP concentration (Bonferroni's test, $p < 0.05$; Table 1). For each AgNP concentration, accumulation of Ag in leaves was higher at 23 than 10 or 16 °C, whereas the accumulation of Ag in leaves treated with $AgNO_3$ was higher at 10 °C than for other temperatures (Bonferroni's test, $p < 0.05$).

After 5 d of animal feeding, the accumulation of Ag in the animal body not exposed to AgNPs or $AgNO_3$ was below the detection limit (<0.3 ng mg^{-1} of wet mass; Table 1). Animals accumulated more Ag when leaves were treated with increasing AgNP concentrations at 10 and 23 °C (Bonferroni's test, $p < 0.05$). After the recovery period, no Ag was detected in the animal body (<0.3 ng mg^{-1} of wet mass) at any temperature (not shown).

TABLE 1: Total silver concentration on microbially colonized leaves pre-exposed to silver nanoparticles or $AgNO_3$ after 21 d of experiment and total silver concentration in the body of *Limnephilus* sp. fed on control and treated leaves, during 5 d at 10, 16, and 23 °C[a]

Temperature	Treatment	mg L^{-1}	Total [Ag] in leaves (μg g^{-1} of dry mass)	Total [Ag] in animal body (ng mg^{-1} of wet mass)
10 °C	Control	0	<5	<0.3
	AgNPs	5	66 ± 2	<0.3
		10	92 ± 2	0.75 ± 0.25
		25	242 ± 153	5.78 ± 0.05
	$AgNO_3$	1	110 ± 22	0.34 ± 0.03
16 °C	Control	0	<5	<0.3
	AgNPs	5	208 ± 31	6.16 ± 1.83
		10	80 ± 10	4.35 ± 1.80
		25	495 ± 107	4.53 ± 1.68
	$AgNO_3$	1	64 ± 12	<0.3
23 °C	Control	0	<5	<0.3
	AgNP	5	241 ± 26	<0.3
		10	143 ± 35	1.09 ± 0.20
		25	592 ± 67	3.35 ± 1.62
	$AgNO_3$	1	59 ± 20	<0.3

[a]Mean ± standard deviation, $n = 3$.
AgNP = silver nanoparticle.

Acute lethality tests

Exposure of invertebrate shredders to AgNPs or $AgNO_3$ had a significant effect on survival (ANOVA, $p < 0.05$). The 96-h LC20 and LC50 values (95% CI) for AgNPs were 7.6 (4.2–10.4) and 11.5 (8.2–18.3) mg L^{-1}, respectively. The 96-h LC20 and LC50 values (95% CI) for $AgNO_3$ were 1.6 (0.4–3.1) and 4.7 (2.5–24.4) mg L^{-1}, respectively.

Leaf consumption by invertebrate shredders

In controls, the average consumption rates of alder leaves by the shredder were 0.062 ± 0.005 (10 °C), 0.066 ± 0.011 (16 °C), and 0.101 ± 0.006 (23 °C) g leaf dry mass g^{-1} animal wet mass d^{-1} (Figure 1A). Consumption rates were significantly affected by temperature and Ag concentration (2-way ANOVA, $p < 0.0001$ and $p = 0.0050$, respectively), and no interaction between factors was found (2-way ANOVA, $p = 0.2789$). The increase in temperature stimulated the consumption rate of the shredder, particularly when animals were exposed to leaves treated with 5 mg L^{-1} AgNPs (23 °C) and 10 mg L^{-1} AgNPs (16 °C; Bonferroni's test, $p < 0.0001$; Figure 1A). Leaf consumption was not significantly affected by exposure to AgNPs or $AgNO_3$ at 10 °C (Bonferroni's test, $p > 0.05$).

After the feeding experiment in the presence of contaminants, animals were recovered from nano and ionic stress and allowed to feed on untreated leaves at the tested temperatures. In control, the average consumption rates by the shredder were 0.061 ± 0.007 (10 °C), 0.054 ± 0.013 (16 °C), and 0.134 ± 0.016 (23 °C) g leaf dry mass g^{-1} animal wet mass d^{-1} (Figure 1B). In the recovery experiment, temperature and Ag concentrations had significant effects on leaf consumption rates (2-way ANOVA, $p < 0.0001$ and $p = 0.0084$, respectively), but no interaction was found between factors (2-way ANOVA, $p = 0.4061$). Consumption rates by the shredders were stimulated at 16 °C when animals were recovered from AgNP or $AgNO_3$ treatments (Bonferroni's test, $p < 0.05$). However, leaf consumption by the shredders did not differ from the control after animals recovered from AgNPs or $AgNO_3$ at 10 or 23 °C (Bonferroni's test, $p > 0.05$; Figure 1B).

Activity of antioxidant and neuronal enzymes

In the exposure feeding experiment, CAT activity in the animal body in controls was 49.6 ± 2.9 (10 °C), 57.4 ± 46.9 (16 °C), and 46.9 ± 27.7 (23 °C) μmol min^{-1} mg^{-1} of protein (Figure 2A). The interaction between temperature and concentration was significant (2-way ANOVA, $p < 0.0001$), but CAT responses were mainly attributable to the Ag concentration (2-way ANOVA, $p = 0.0006$). At 10 °C, CAT activity was stimulated only when the animals were fed on leaves treated with the highest AgNP concentration (Bonferroni's test, $p < 0.05$), whereas at 23 °C CAT activity was stimulated in animals fed on leaves treated with $AgNO_3$ (Bonferroni's test, $p < 0.05$).

In controls, the activity of SOD was 141.9 ± 8.1 (10 °C), 150.5 ± 63.7 (16 °C), and 133.4 ± 29.8 (23 °C) μmol min^{-1} mg^{-1} of protein (Figure 2C). The activity of SOD did not differ

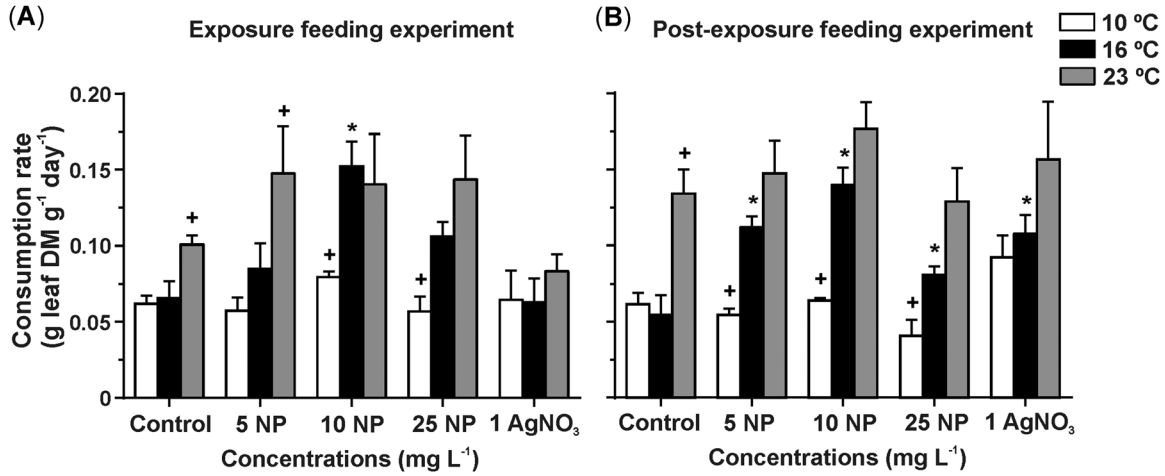

FIGURE 1: Consumption of alder leaves treated with silver nanoparticles (AgNPs) or $AgNO_3$ by the shredder *Limnephilus* sp. during 5 d (exposure feeding experiment, **A**) and after 3 d in clean water and fed on untreated leaves (postexposure feeding experiment, **B**) at 10 °C (white bars), 16 °C (black bars), and 23 °C (gray bars). *Significant effects of AgNPs or $AgNO_3$ compared to control at each temperature; $^{+}$significant effect of temperature at each AgNP or $AgNO_3$ concentration (2-way analysis of variance, Bonferroni's test, $p < 0.05$). Mean ± standard error of the mean ($n = 3$). DM = dry matter.

between treatments at higher temperatures, but at 10 °C it was stimulated when animals were fed on leaves treated with 10 mg L^{-1} AgNPs (Bonferroni's test, $p < 0.05$; Figure 2C). Temperature and Ag concentrations had significant effects on SOD activity (2-way ANOVA, $p = 0.0014$ and $p = 0.0011$, respectively), but no interaction between factors was found (2-way ANOVA, $p = 0.1343$).

In controls, the activity of GST was 5.8 ± 0.6 (10 °C), 5.5 ± 2.5 (16 °C), and 16.5 ± 6.3 (23 °C) μmol min^{-1} mg^{-1} of protein (Figure 2E). Temperature and Ag concentrations had significant effects on GST activities (2-way ANOVA, $p = 0.0126$ and $p = 0.0023$, respectively), and the interaction between factors was significant ($p < 0.0001$). The activity of GST was stimulated at 10 and 16 °C in animals fed on leaves treated with 5 and 25 mg L^{-1} AgNPs (Bonferroni's test, $p < 0.05$), respectively.

The activity of AChE was significantly affected by temperature and Ag concentrations (2-way ANOVA, $p < 0.0001$), and there was an interaction between these factors (2-way ANOVA, $p < 0.0001$). At 16 °C, AChE activity was inhibited in animals that fed on leaves treated with AgNPs, whereas at 10 °C AChE activity was stimulated in animals fed on leaves treated with AgNPs (25 mg L^{-1}) or $AgNO_3$ (Figure 2G).

In the postexposure feeding experiment, the release of animals from nano and ionic stress for 3 d led to different responses of the enzyme activities, mainly attributable to differences in temperature (2-way ANOVA, $p < 0.0001$, for all enzymes analyzed). Following 3 d of recovery from Ag exposure, CAT activity was significantly affected by the interaction between temperature and Ag concentration (2-way ANOVA, $p < 0.0001$). The activity of CAT did not differ in animals pre-exposed to treated leaves at 10 °C (Bonferroni's test, $p > 0.05$), but it was stimulated at 23 °C in animals pre-exposed to leaves treated with the highest AgNP concentration (Bonferroni's test, $p < 0.05$; Figure 2B). On the contrary, CAT activity was inibited at 16 °C in animals pre-exposed to leaves treated with $AgNO_3$ and 10 mg L^{-1} AgNPs. The activity of SOD was affected by the interaction between temperature and AgNP concentration (2-way ANOVA, $p < 0.0001$). The activity of SOD was stimulated at 23 °C in animals pre-exposed to leaves treated with 10 and 25 mg L^{-1} AgNPs (Bonferroni's test, $p < 0.05$; Figure 2D) and at 16 °C in animals pre-exposed to leaves treated with 5 mg L^{-1} AgNPs. The activity of GST was significantly affected by temperature but not by AgNPs or $AgNO_3$ (2-way ANOVA, $p < 0.0001$ and $p = 0.5744$, respectively; Figure 2F). No interactions between factors were found on the GST activity (2-way ANOVA, $p = 0.9666$). In the postexposure feeding experiment, there was an interaction between temperature and Ag concentration (2-way ANOVA, $p < 0.0001$), but only the effects of temperature on AChE responses were significant (2-way ANOVA, $p < 0.0001$). The activity of AChE was inibited in animals pre-exposed to 10 and 25 mg L^{-1} AgNP-treated leaves at 10 and 23 °C, respectively, and at 16 °C in animals pre-exposed to $AgNO_3$-treated leaves (Bonferroni's test, $p < 0.05$).

Relationships between stress biomarkers, temperature, and toxicants

The relationships between oxidative and neuronal stress responses after exposure and postexposure feeding experiments, total Ag in the animal body, temperature, and concentrations of AgNPs and $AgNO_3$ were revealed by PCA (Figure 3). Principal components 1 and 2 (PC1 and PC2) were the variables produced by the PCA that explained 30.2 and 18.4% of the total variance, respectively (Figure 3). The principal components, eingenvalues, and eingenvectors are presented in Supplemental Data, Table S3. The PCA showed a clear segregation between temperatures along the PC1 axis

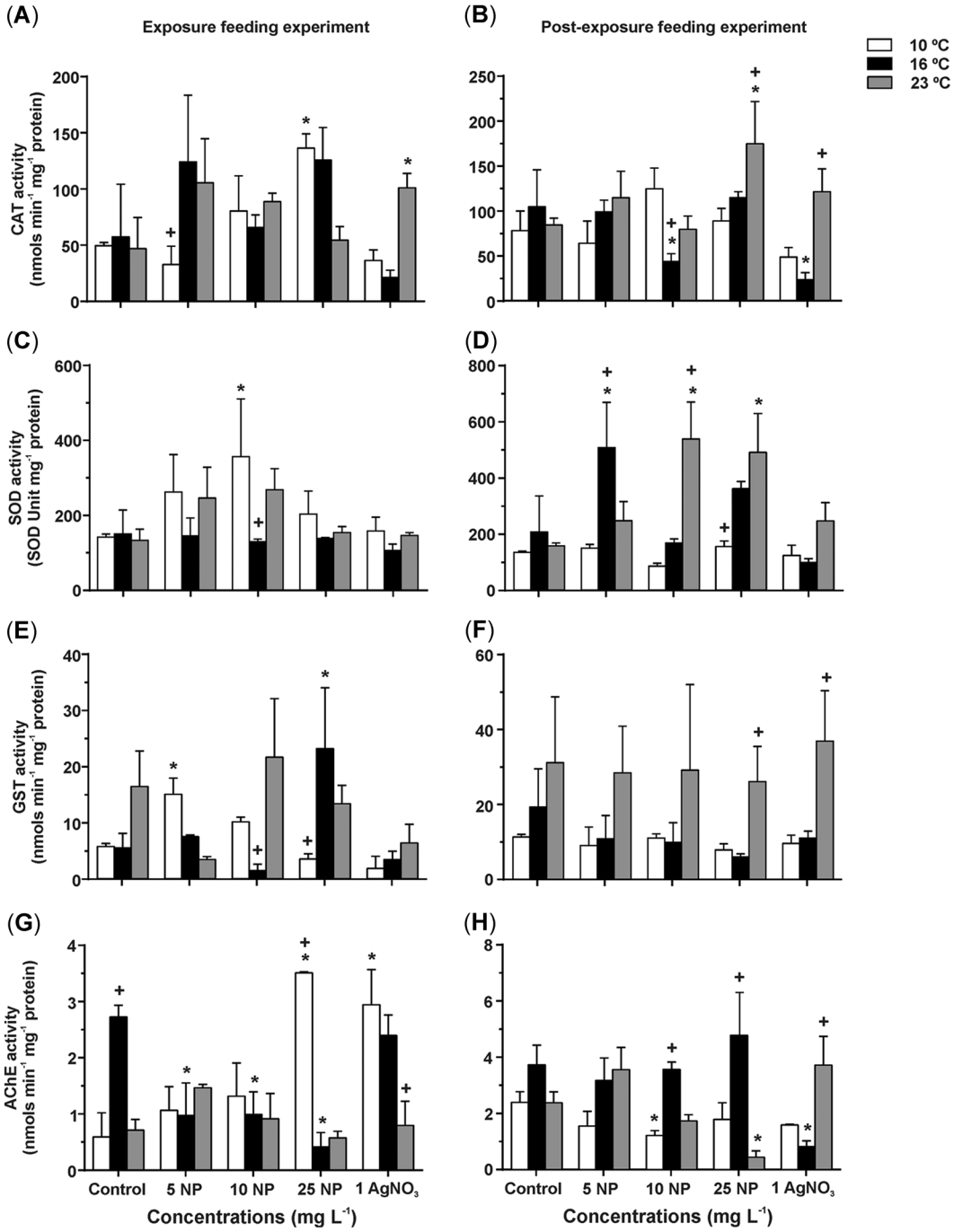

FIGURE 2: Activities of catalase (**A**, **B**), superoxide dismutase (**C**, **D**), glutathione *S*-transferase (**E**, **F**), and acetylcholinesterase (**G**, **H**) in the shredder *Limnephilus* sp. fed on alder leaves treated with silver nanoparticles (AgNPs) or $AgNO_3$, during 5 d (exposure feeding experiment, **A**, **C**, **E**, **G**) and after 3 d on clean water and fed on untreated leaves (postexposure feeding experiment, **B**, **D**, **F**, **H**) at 10, 16, and 23 °C. *Significant effects of AgNPs or $AgNO_3$ compared to control at each temperature; $^{+}$significant effect of temperature at each AgNP or $AgNO_3$ concentration (2-way analysis of variance, Bonferroni's test, $p < 0.05$). Mean ± standard error of the mean ($n = 3$). AChE = acetylcholinesterase; CAT = catalase; GST = glutathione *S*-transferase; SOD = superoxide dismutase.

based on biomarker responses (Figure 3). The activity of AChE in the exposure experiment was positively associated with the lowest temperature (10 °C) along PC1, whereas the activities of most oxidative stress enzymes were associated with higher temperatures (Figure 3; Supplemental Data, Table S3). Apart from GST, the activities of antioxidant enzymes after the feeding experiments were positively associated with the total Ag accumulated in the animal body along PC2 (Figure 3; Supplemental Data, Table S3).

DISCUSSION

The present study shows interactive effects of temperature and AgNPs on the performance of freshwater invertebrates

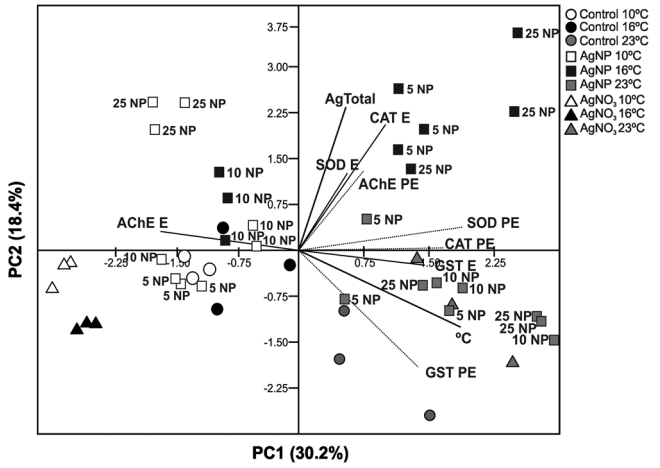

FIGURE 3: Principal component analysis of overall responses of stress biomarkers in the shredder *Limnephilus* sp. fed on alder leaves treated with silver nanoparticles or $AgNO_3$, during 5 d (exposure feeding experiment) and after 3 d on clean water and fed on untreated leaves (postexposure feeding experiment), at different temperatures (10, 16, and 23 °C). AChE = acetylcholinesterase; AgNP = silver nanoparticle; CAT = catalase; E = exposure; GST = glutathione *S*-transferase; NP = nanoparticle; PC = principal component; PE = postexposure; SOD = superoxide dismutase.

(*Limnephilus* sp.) and provides relevant information for understanding the impacts of the increased use of nanomaterials under warming temperatures. A stimulation of plant litter consumption by the shredders was observed with increased temperature and in the presence of contaminants, presumably to supply additional energy to the animals to face the stressors. If augmented plant litter consumption will be observed in natural ecosystems, it may lead to a fast depletion of food resources and to changes in the carbon and energy fluxes in freshwaters.

In the present study, the shredder *Limnephilus* sp. survived under acute exposures to high concentrations of AgNPs or $AgNO_3$ (LC50 = 11 and 5 mg L^{-1}, respectively, acute lethality tests) compared to other invertebrates in previous studies (*Gammarus fossarum*, LC50 = 0.8–1 mg L^{-1} AgNPs [Mehennaoui et al. 2016]; LC50 = 2.2 µg L^{-1} $AgNO_3$ [Arce-Funck et al. 2013]). The lower toxicity of these contaminants to *Limnephilus* compared to other organisms can be partially explained by the adsorption of contaminants, such as metals or nanometals, to the animal case (Batista et al. 2012; Pradhan et al. 2015). This indicates that animals with cases can be better prepared to face contaminants because they have an extra barrier that reduces direct contact with the stressors.

As expected, leaf consumption by shredders was stimulated by the rise in temperature. This fact has been reported in invertebrate detritivores (*G. pseudolimnaeus*: Galic and Forbes 2017; *Limnephilus* sp.: Batista et al. 2012; *Schizopelex* sp.: Silva et al. 2018). Moreover, we expected a recovery in the feeding activity of the shredder after the recovery from the nano and ionic Ag stress, which was not the case (Figure 1B). A rise in temperature augments the metabolic rates of organisms, probably leading to increases in development rates, respiration, and growth, together with increased consumption and excretion rates (Dallas and Ross-Gillespie 2015). Increased metabolic rates imply more energy output (Chung and Suberkropp 2009), meaning that the shredders in the present study would have consumed more leaves to compensate for the energy lost with the metabolism acceleration.

The effects on consumption rates in the present study were also related to the presence of AgNPs in food (leaf litter). Similarly to what was observed after exposure to high temperatures, toxicants can trigger energy-dependent defense mechanisms (e.g., detoxification), changing animal fitness through a reduction in growth and reproduction (Kooijman et al. 2009). Exposure to toxicants can alter the structure of fungal communities on decomposing leaves (Batista et al. 2012, 2017b; Pradhan et al. 2015), affecting leaf quality and palatability for shredders and reducing their consumption rates, which was not the case in the present study. In the present study, microbially colonized leaves were offered to the animals; this may have contributed to the short metal effects on leaf consumption by shredders. The species richness was higher in microcosms exposed to 10 °C, but some fungal species appeared to have a preference for certain temperatures, as well as being present at certain AgNP and $AgNO_3$ concentrations (see Batista et al. 2017a).

In the present study, the activity of antioxidant enzymes in the shredders after dietary exposure to AgNPs or $AgNO_3$ changed with temperature and the concentration of AgNPs or $AgNO_3$, supporting the role of these enzymes in triggering the defense against oxidative stress. In the feeding experiment, CAT and SOD were stimulated in animals feeding on leaves treated with a high concentration of AgNPs at 10 °C (Figure 2A, C), suggesting increased intracellular accumulation of ROS. The raised GST activity observed in the animals feeding on leaves treated with the higher AgNP concentration at 16 °C (Figure 2E) further indicated that animals were under oxidative stress (Wu et al. 2017). A higher GST activity implies a greater detoxification capacity or conjugation of oxidative products. Under these conditions, neuronal functions were also compromised, as suggested by the inhibition of AChE activity in the shredders (Figure 2G). Metal-based NPs and ionic form of metals have been reported to affect the activities of AChE in aquatic organisms (Pradhan et al. 2016). Moreover, AgNPs have been reported to inhibit AChE in a dose-dependent manner (Šinko et al. 2014).

After recovery from the toxicants, the enzymatic activities (GST, AChE) in the shredders were generally higher at 16 and 23 °C (Figure 2F, H). Our findings indicate that the antioxidant enzymes respond to temperature, probably because of the induction of ROS production, and are consistent with a positive relationship between thermal and oxidative stress responses of antioxidant enzymes at least up to a certain threshold, as found in crabs exposed to AgNPs at 28 °C (Walters et al. 2016).

The total Ag in the animal body after feeding on leaves treated with AgNPs or $AgNO_3$ was low compared to the concentrations in treated leaves. Several authors have reported Ag accumulation in freshwater invertebrates after dietary exposure to metals (daphnia fed on algae for 48 h: McTeer et al. 2014; Ribeiro et al. 2017). Probably during the feeding exposure, Ag

leached out from the leaves into the water, and the high consumption rates observed in the present study might have led to an increase in the excretion rate, explaining the low concentrations of Ag in the animal body after 5 d. Moreover, our inability to detect Ag in the animal body after stress release suggests that animals were able to eliminate Ag by feces excretion, as observed by others (Zhao and Wang 2012; Andreï et al. 2016).

In the present study, enzymatic activities were correlated with the total Ag accumulated in the animal body (except for GST). Exposure to leaves treated with AgNPs triggered responses at the cellular level despite the low concentrations of Ag in the animal body, indicating that the animals were under oxidative stress. Our findings further support the role of antioxidant enzymes as early indicators of AgNP stress. Exposure to AgNP-contaminated food stimulated the feeding activity of shredders and may have triggered AgNP elimination by the production of fine particulate organic matter, including feces. This might have negative effects on other detritivores, such as filter-feeders and collector-gatherers, with consequences to detritus-based food webs in freshwaters.

CONCLUSIONS

The increase in temperature led to a stimulation in plant litter consumption by the shredder *Limnephilus* sp. However, the higher consumption rates were also related to the contamination of food by AgNPs or $AgNO_3$. The observed absence of negative effects on shredder consumption rates can be misleading because results from the enzymatic activities indicate that AgNPs induced oxidative and neuronal stress in the animals. The effects of toxicants on the consumption rates and enzymatic activities were clearly altered by temperature, highlighting the importance of assessing the effects of increased temperature on aquatic organisms that are already under stress.

The concentrations used in the present study were higher than those expected in the most extreme environmental settings, such as direct wastewater exposures. Therefore, the present study should be considered a proof of concept of potential environmental effects, with further study required to assess the potential for actual effects in streams. As a whole, the present study brings new insights to the understanding of environmental impacts associated with exposure to AgNPs via food, providing evidence that AgNPs and increased temperature affect the activity of invertebrate shredders, with potential implications to other trophic levels in aquatic detritus-based food webs.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4738.

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