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► To cite this version:

Jennifer Andreï, François Guéroid, Jonathan Bouquerel, Simon Devin, Kahina Mehennaoui, et al.. Assessing the effects of silver nanoparticles on the ecophysiology of *Gammarus roeseli*. *Aquatic Toxicology*, 2023, 256, 10.1016/j.aquatox.2023.106421 . hal-04034863

HAL Id: hal-04034863

<https://hal.univ-lorraine.fr/hal-04034863>

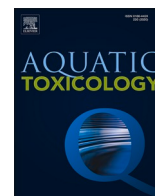
Submitted on 17 Mar 2023

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Assessing the effects of silver nanoparticles on the ecophysiology of *Gammarus roeseli*

Jennifer Andreï^a, François Guérolde^a, Jonathan Bouquerel^a, Simon Devin^a,
Kahina Mehennaoui^{a,b}, Sebastien Cambier^b, Arno C. Gutleb^b, Laure Giambérini^{a,c},
Sandrine Pain-Devin^{a,*}

^a Université de Lorraine, CNRS, LIEC, F-57000 Metz, France

^b Environmental Research and Innovation (ERIN) Department, Luxembourg Institute of Science and Technology (LIST), 41, rue du Brill, L-4422 Belvaux, Luxembourg

^c International Consortium for the Environmental Implications of Nanotechnology (iCEINT), Aix en Provence, France

ARTICLE INFO

Keywords:

Gammarus roeseli
Silver
Nanoparticles
Ecophysiology
Oxygen consumption
Energetic metabolism

ABSTRACT

Being part of the macrobenthic fauna, gammarids are efficient indicators of contamination of aquatic ecosystems by nanoparticles that are likely to sediment on the bottom. The present study investigates the effects of silver nanoparticles (nAg) on ecophysiological functions in *Gammarus roeseli* by using a realistic scenario of contamination. Indeed, an experiment was conducted during 72 h, assessing the effects of 5 silver nAg from 10 to 100 nm diluted at concentrations of maximum $5 \mu\text{g L}^{-1}$ in a natural water retrieved from a stream and supplemented with food. The measured endpoints in gammarids were survival, silver concentrations in tissues, consumption of oxygen and ventilation of gills. Additionally, a set of biomarkers of the energetic metabolism was measured. After a 72-h exposure, results showed a concentration-dependent increase of silver levels in *G. roeseli* that was significant for the smallest nAg size (10 nm). Ecophysiological responses in *G. roeseli* were affected and the most striking effect was a concentration-dependent increase in oxygen consumption especially for the smallest nAg (10 to 40 nm), whereas ventilation of gills by gammarids was not changed. The potential mechanisms underlying these findings are discussed. Thus, we demonstrated the very low exposure concentration of $0.5 \mu\text{g L}^{-1}$ for the small nAg size led to significant ecophysiological effects reinforcing the need to further investigate subtle effects on nanoparticles on aquatic organisms.

1. Introduction

The development of nanotechnologies is leading to a wide use of engineered nanoparticles for many industrial applications and in consumer products (Vance et al., 2015). Among them, silver nanoparticles (nAg) are of particular interest because of their broad spectrum of antimicrobial and optical properties. They are consequently among the most exploited for applications such as biomedical, food packaging, cosmetics, textiles, coating or paints (Addo Ntim et al., 2015; Durán et al., 2007; Keller et al., 2013; Künniger et al., 2014; Marambio-Jones and Hoek, 2010). Several studies pointed out the risk of potential nAg release in the environment throughout their entire life cycle, suggesting emission during their manufacturing, their incorporation into products, and especially during their use, disposal and recycling (Benn and Westerhoff, 2008; Gottschalk and Nowack, 2011; Kaegi et al., 2010;

Keller et al., 2013; Sun et al., 2014). The techniques currently available, as well as the lack of practical approaches make it difficult to carry out routine and large-scale quantification of nanoparticle emissions and concentrations in the environment (McGillicuddy et al., 2017), making mathematical modelling the preferred tool for predicting the environmental concentrations. The predicted environmental concentrations (PEC) for nAg in the European surface water were calculated to be in the lower ng L^{-1} range (Gottschalk et al., 2009; Gottschalk and Nowack, 2011, Sun et al., 2014). Even so, the scientific community agrees that despite the estimation of low PEC, numerous uncertainties are remaining about their actual concentrations in the environment, about their fate and effects, especially in the current context of increasing use of nAg. Therefore, besides enhancing the ability to detect and quantify them in natural complex matrices (for instance for nAgs: Furtado et al., 2016), one of the main priorities is to address an appropriate risk

* Corresponding author.

E-mail address: sandrine.devin@univ-lorraine.fr (S. Pain-Devin).

<https://doi.org/10.1016/j.aquatox.2023.106421>

Received 27 October 2022; Received in revised form 13 January 2023; Accepted 5 February 2023

Available online 10 February 2023

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assessment for freshwater ecosystems by using environmentally realistic exposure concentrations and media (Tiede et al., 2009; Mc Gillicuddy et al., 2017; Bundschuh et al., 2018).

The risk of potentially adverse effects in aquatic environments may be associated with the nanoparticulate form of silver, and/or, with the release of dissolved silver by oxidative dissolution which strongly depends on the physico-chemical characteristics of the water and on the nAg itself, namely its size and its surface coating (Lee et al., 2012; Liu and Hurt, 2010; Peretyazhko et al., 2014; Zhang et al., 2016; Mehennaoui et al., 2018). Silver is recognised as highly toxic for aquatic organisms even at very low concentrations (Arce Funck et al., 2013; Ratte, 1999). Many studies showed that nAg exert biological effects that could not be attributed to the release of dissolved silver, suggesting a real toxicity of the nanoparticulate form (Andrei et al., 2016; Chae et al., 2009; Gomes et al., 2014; Griffitt et al., 2008). Silver nanoparticles were found to induce DNA damages, cytotoxicity, oxidative stress, antioxidant and antioxidant responses in aquatic organisms (Farkas et al., 2011; Lapresta-Fernandez et al., 2012; Rocha et al., 2015; Vale et al., 2016; Mehennaoui et al., 2021).

Freshwater gammarids are now widely used in ecotoxicological surveys because they are widespread in various freshwater systems, easy to handle during laboratory testing and especially because they allow assessing effects of pollutants both on the organism itself and on the ecosystem functioning (Guerlet et al., 2008; Dedourge-Geffard et al., 2009; Vellinger et al., 2013; Zubrod et al., 2015). As part of the benthic macro-fauna, gammarids are of particular interest for investigating the potentially adverse effects of nanoparticles, that are likely to homo- or hetero-aggregate and so, that are likely to reach the bottom of aquatic systems where gammarids preferentially live. The laboratory exposure of gammarids to various nAgs showed different kind of effects from molecular to functional levels. In *Gammarus fossarum*, effects were reported on the gene expression, on the development of the digestive lysosomal system, on the locomotor activity (Mehennaoui et al., 2016; 2021) and on the energy assimilation (Lüderwald et al., 2019). Energy assimilation and locomotor activity were also affected by nAgs exposure in *Gammarus roeseli* (Andrei et al., 2016). Considering this and to get further insights in ecophysiological effects of nAgs, the present work used *G. roeseli* as a model.

In contaminated aquatic ecosystems, one of the major routes of pollutant entry in the biota are the gills because there are in direct contact with the pollutant and therefore may be primarily impacted. Effects of nAg on the gills and on the respiratory process are well documented for fish (Bilberg et al., 2010; Cowart et al., 2011; Farkas et al., 2011; Griffitt et al., 2012; Handy et al., 2011). It has been shown that nAg could increase the gill thickness and reduce oxygen transport, leading to an internal organism hypoxia (Bilberg et al., 2010; Griffitt et al., 2012; Srinonate et al., 2015). More specifically, studies conducted in gammarids reported effects of metals on ventilation (Arce Funck et al., 2013; Felten et al., 2008) or on electron transport system activity (ETS, Schmidlin et al., 2015). Recently sublethal effects of nAg in *G. fossarum* were reported and the authors hypothesized that uptake in the gills may be related to further alteration in energy metabolism (Mehennaoui et al., 2021). Here we hypothesized that nAg may affect the ecophysiology of *G. roeseli* by altering respiratory process and in consequence energy supply. In this context, we investigated ventilation activity and oxygen consumption in *G. roeseli*. To look at energy supply, we measured ETS activity indicating the use of oxygen in mitochondria to complete the production of ATP and we assessed the concentrations of triglycerides and proteins as they are substrates for cells to produce ATP (Garaud et al., 2016; Sroda and Cossu-Leguille, 2011).

The present study provides a realistic scenario of contamination in a freshwater ecosystem. Gammarids were exposed to very low range of nAg concentrations in natural water retrieved from a stream and supplemented with food. The objectives were first to evaluate the effects of nAg on respiratory and energy supply processes and to determine whether this set of energetic endpoints could be an effective tool to

assess the effects of nAg in gammarids. Finally, we also aimed to determine whether the effects were dependent on the concentration and the size of nAg.

2. Material and methods

2.1. Silver nanoparticle characterization

Five sizes of spherical nAg (10, 20, 40, 60 and 100 nm) suspended in a citrate buffer stabilizer capping agent were purchased from Nanocomposit Europe (Prague, Czech Republic). The stock solutions, with a silver concentration of 0.02 mg mL^{-1} were stored in the dark at 4°C . Hydrodynamic diameters of the tested nAg were assessed by using a particle tracker (NanoSight 500, Malvern Instrument Ltd, UK). The nAg size distribution was also characterized in the natural water from La Méholle used for the exposure. Those measurements were performed with the 5 nAg sizes at $5 \mu\text{g L}^{-1}$ but also at a higher optimal concentration of $100 \mu\text{g L}^{-1}$.

2.2. Biological material collection and maintenance

The water used during the experiment was sampled in a reference stream La Méholle (Void-Vacon, N-E France, see SI Table S1 for chemical characteristics). The water was filtered using glass microfibre filters (90 mm diameter, pore size $1.2 \mu\text{m}$, Whatman®) to remove suspended particles and stored at 12°C in the dark (48 h before the start of the experiment).

Gammarus roeseli (Crustacea, Amphipoda; Gervais, 1835) were collected from the river La Nied (Remilly, N-E France). Male gammarids were selected. They were acclimated for 3 days by gradual replacement of their water of origin by the test water (La Méholle). Then, they were let in the La Méholle water during 6 to 10 days before being exposed. During all the acclimation period, they were fed *ad libitum* with alder leaves (*Alnus glutinosa*) with a 16:8 h light:dark cycle.

During the experiment, the gammarids were fed with senescent dried alder leaves. Leaf-disks of 16 mm diameter were cut, placed in nylon bags (mesh-size $500 \mu\text{m}$) and immersed in the La Méholle stream to allow for microorganisms conditioning. After 12 days, the disks were retrieved and dried during 48 h at 105°C before use.

2.3. Exposure design

The exposures were conducted under controlled conditions [$12.1 \pm 0.2^\circ\text{C}$; 16:8 h light: dark cycles]. Test solutions were prepared by diluting the nAg stock suspension (0.02 mg mL^{-1}) with filtered and oxygen saturated water from La Méholle to obtain nominal concentrations of 0, 0.5, 2.5 and $5 \mu\text{g L}^{-1}$.

For each treatment (5 nAg sizes 10, 20, 40, 60 and 100 nm and 4 concentrations 0, 0.5, 2.5 and $5 \mu\text{g L}^{-1}$), a 48 h silver saturation of polypropylene microcosms was applied with the relevant exposure concentrations (4 replicates/condition). Then, the preconditioning solution was replaced by 300 mL of exposure solution per microcosm (each containing 4 alder discs). Ten gammarids per replicate sample were then exposed for 72 h. Finally, the experimental design can be summarised as follows: 5 nAg sizes x 4 concentrations x 4 replicate samples x 10 gammarids.

The saturation and exposure experiments were started with a delay of 24 h between each nAg size. In each microcosm ($n=80$), 2 samples of water (4 mL) were removed after 72 h to determine the final $[\text{Ag}]_{\text{total}}$ and $[\text{Ag}]_{\text{dissolved}}$. Samples were taken from the middle of the water column taking care not to remove the particles that have settled at the bottom. Dissolved Ag was separated from the nanoparticulate form using Amicon® ultra-15 centrifugal filter devices (porosity 3 kDa, centrifuged for 40 min at $5,000 \times g$). Concentrations were measured using Atomic Absorption Spectroscopy (AAS, Detection Limit: $0.1 \mu\text{g L}^{-1}$) analysis after 1% HNO_3 acidification as previously reported (Andrei

et al., 2016). The percentage of dissolution was then calculated as the ratio of dissolved [Ag] to total [Ag]. At the end of the experiment, gammarids were retrieved, used for analyses or stored at -80°C before treatment.

2.4. Biological endpoints

2.4.1. Survival

Every 24 h, the number of living *G. roeseli* was counted and the dead organisms were removed.

2.4.2. Silver concentration in gammarids

After 72 h of nAg exposure, a pool of 3 organisms per replicate sample ($n=4$ pools) was randomly collected and stored at -80°C until analysis. Then, they were dried during 48h at 105°C and weighed. The samples were digested in nitric acid (HNO_3 , LGC Standards) and hydrogen peroxide (H_2O_2 , Fisher scientific). The mineralization was performed with a microwave reaction system before silver analysis by inductively coupled plasma-mass spectrometry (ICP-MS, XSERIES 2ⁱ, ThermoFisher Scientific). Silver concentrations in gammarids were expressed as $\mu\text{g g}^{-1}$ of dry mass.

2.4.3. Respiration and energetic markers

2.4.3.1. Oxygen consumption. Assessment of gammarids respiration were performed with a respirometer Unisense Microrespiration System (UNISENSE A/S, Aarhus Denmark) which is able to measure the oxygen consumption in real time in small volumes with very low detection limits. At the end of the experiment, one gammarid per replicate sample ($n=4$) was gently collected and disposed in a 2 mL chamber containing uncontaminated La Méholle water, filtered and oxygen saturated. The decrease in oxygen concentration in the chamber corresponds to the consumption by gammarids. It was measured with a microsensor during 30 minutes. Two simultaneous analyses on two gammarids were achieved by using 2 different chambers and 2 microsensors. The rates of oxygen consumption were calculated by using linear regression performed in the regular portion of the curve. The results were expressed as $\mu\text{g O}_2$ per mg of gammarid dried mass per hour.

The correlation between oxygen consumption and silver concentrations in gammarids was tested with a linear model.

2.4.3.2. Ventilation. Measurements of gammarids ventilation were performed by counting the pleopod beating frequency according to Felten et al. (2008). Pleopods are appendages in constant beating favouring oxygen uptake by the gills (Sutcliffe, 1984). After measurement of the oxygen consumption, the gammarids ($n=4$) were removed from the analytic chamber and transferred individually in a well of a 24-wells microplate containing 2 mL of the uncontaminated filtered La Méholle water. After 30 seconds of acclimation, video recordings of the pleopod beating were performed during 30 seconds. The gammarids were then retrieved and stored at -80°C before energetic biomarkers analysis. The videos were analysed several days after by counting the number of beating during 10 seconds and 3 times for a same organism. The results were calculated as the ratio of the pleopod beat min^{-1} in contaminated gammarids to that in control organisms and expressed as percentage.

2.4.3.3. Energetic biomarkers. Gammarids used for the oxygen consumption and the ventilation measurements and then stored at -80°C ($n=4$) were defrosted at 4°C , gently dried on an absorbent paper and weighed. They were then immersed in a phosphate buffer (50 mM, pH 7.6 supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM L-serine borate mixture as protease inhibitors) with a 4/1 volume/weight ratio before to be mechanically grounded during 2 rounds of 30 seconds with a beadbeater (Retsch MM301) (Garaud et al., 2016; Sroda

and Cossu-Leguille, 2010). The homogenates were then centrifuged for 5 minutes at $250 \times g$ and 4°C to remove the cuticle fragments. The supernatants were recovered for biomarker measurements with the automated colorimetric methods and the protocols developed by Garaud et al. (2016) for the Konelab 20-XTi (Thermo Scientific).

First, a Scal bovine-based serum purchased from Thermo-Scientific was used to perform calibrations curves for protein and triglyceride analyses. The concentrations were then measured by using ready-to-use reagents and protocols established by Garaud et al. (2016) for *Dreissena polymorpha*, and adapted for gammarids. Results were expressed in $\text{mg. g fresh weight}^{-1}$ (mg. g FW^{-1}).

Measurements of the electron transport system (ETS) mitochondrial activity were also performed with the same supernatant sample following the method described in Garaud et al. (2016) that was adapted from Owens and King (1975) and De Coen and Janssen (1997) and automated on the Konelab. The results were expressed in $\mu\text{mol O}_2. \text{g fresh weight}^{-1}.\text{h}^{-1}$ ($\mu\text{mol O}_2.\text{g FW}^{-1}.\text{h}^{-1}$).

2.5. Statistical analysis

Statistical analyses were performed using R 3.1.0 (R Development Core Team, 2014). Parametric conditions before the one-way ANOVA were met for silver concentrations in gammarids but not for those in water, so respectively, one-way ANOVA and Kruskal-Wallis ANOVA analyses were performed. For oxygen consumption data, a one-way ANOVA was performed to test the difference between the 5 control conditions, and because the measurements were performed with a 24 h delay between each size conditions, a one-way ANOVA was conducted for each of them ($n=16$). When significant differences were evidenced, post-hoc tests were performed (Tukey HSD for ANOVA, kruskalmc function of the pgirmess library for the non-parametric data). To assess general patterns of respiration and energetic markers response to nAg size and concentration, we performed a linear discriminant analysis (function lda of the library MASS and discDA of the library DiscrMiner). The overall significance of the model was based on the Wilk's statistic following a MANOVA. The desDA function of the DiscrMiner library was used to define whether each biomarker significantly contributes to the discrimination of the exposure conditions (nAg size and concentration).

3. Results

3.1. Silver nanoparticle characterization

The hydrodynamic diameters of the nAg measured in deionized water were consistent with the manufacturer measurements (Table 1). The particle size distribution in the samples of La Mehollé spiked with $5 \mu\text{g L}^{-1}$ of nAg were similar to particle size distribution curve of the La Mehollé river without any nAg indicating that concentrations were under the limit of detection. However, the nanoparticle characterization at $100 \mu\text{g L}^{-1}$ of the 10, 20, 40, 60 and 100 nm nAg in La Mehollé water showed particles with hydrodynamic diameters of 56 ± 3.1 , 84 ± 16.8 , 90 ± 4.7 , 69 ± 6.1 and 88 ± 5.4 nm respectively, indicating that La Mehollé water had modified the nAg size distribution (SI Fig. S1).

Table 1

Hydrodynamic diameters provided by the manufacturer Nanocompositix and hydrodynamic diameters (mean \pm S.D.) measured with a NanoSight 500 (Malvern Instrument Ltd, UK) before the start of the experiment.

nAg sizes	Hydrodynamic diameter (nm)	
	Nanocompositix	Nanosight
10 nm	14.6	14 ± 1.3
20 nm	27.2	24 ± 1.2
40 nm	41.8	46 ± 0.3
60 nm	62.5	58 ± 1.0
100 nm	106.5	92 ± 2.6

3.2. Concentrations of silver in water and in gammarids

At the start of the experiment, the silver initial concentrations in each of the four replicates of nAg test solutions were checked for 0, 0.5, 2.5 and 5 $\mu\text{g L}^{-1}$: <DL, 0.10 ± 0.07 , 1.34 ± 0.18 and 3.56 ± 0.52 respectively.

The total silver concentrations (mean \pm SD) measured in the water at the end of the experiment were summarized in the Table 2. Regardless of the nAg size tested, the total Ag concentrations measured after 72 hours in all conditions were different from each other, except between the control and 0.5 $\mu\text{g L}^{-1}$ (Kruskal-Wallis chi-squared = 55.7805, df = 3, P-value <0.01; Kruskal test, $P=0.05$).

The presence of dissolved silver was monitored and its concentration was under the AAS detection limits of 0.1 $\mu\text{g L}^{-1}$ for all the conditions and the totality of the samples.

The concentrations measured in gammarids exposed to 0.5 $\mu\text{g L}^{-1}$ nAg were never different from the control, regardless of nAg size (Fig. 1). For each nAg size, we observed an accumulation of silver as a function of exposure concentration, but it was not significant except for the 10 nm nAg. Indeed, the smallest nAg showed a 3-fold increase in accumulation at 2.5 $\mu\text{g L}^{-1}$ and an almost 6-fold increase at 5 $\mu\text{g L}^{-1}$ compared to the control.

3.3. Biological endpoints

3.3.1. Survival

Survival rates ranged from 83% to 100% and the exposure to nAg did not induce significant gammarid mortality compared to the control.

3.3.2. Discriminant analysis performed with respiration and energetic responses

The discriminant analysis performed with the data obtained from 5 energetic markers (detailed values in SI Table S2) is presented in the Fig. 2.

The LDA evidenced that energetic markers were able to discriminate between nAg size and concentration (MANOVA, Wilk's $\lambda=0.077$, df=19, $P=5.07 \cdot 10^{-6}$). The first two discriminant functions explained 88% of the overall variance and the error rate remains quite high (72.5%). That could be easily explained by the similarity of responses between the control of each nAg size, leading to numerous misclassifications. The LDA also showed that the smaller is the nAg, the higher is the dispersion according to concentration in the first axis. Finally, the oxygen consumption was sufficient to significantly discriminate between the different exposure conditions ($P=2.18 \cdot 10^{-14}$), while the influence of the other markers was not significant (Ventilation, $P=0.47$; ETS activity, $P=0.86$; triglycerides concentration, $P=0.96$; protein concentration, $P=0.98$).

3.3.3. Oxygen consumption

Significant differences between concentrations were detected for 3 nAg sizes out 5 (Fig. 3): 10 nm (one-way ANOVA, $F=47.55$, df=3, $P<0.001$), 20 nm (one-way ANOVA, $F=19.03$, df=3, $P<0.001$) and 40 nm (one-way ANOVA, $F=8.35$, df=3, $P<0.001$). For the 10 nm conditions, the gammarid oxygen consumptions were significantly different

Table 2

Mean (\pm S.D.) total silver concentration ($\mu\text{g L}^{-1}$) measured after 72 h in water at 4 nominal concentrations (0, 0.5, 2.5 and 5 $\mu\text{g L}^{-1}$) of 5 nAg size (10, 20, 40, 60 and 100 nm). DL: detection limit (0.1 $\mu\text{g L}^{-1}$).

nAg sizes	0 $\mu\text{g L}^{-1}$	0.5 $\mu\text{g L}^{-1}$	2.5 $\mu\text{g L}^{-1}$	5 $\mu\text{g L}^{-1}$
10 nm	< DL	0.28 ± 0.05	1.06 ± 0.22	2 ± 0.33
20 nm	< DL	0.15 ± 0.06	0.98 ± 0.25	3.01 ± 0.93
40 nm	< DL	0.30 ± 0.08	1.88 ± 0.81	4.15 ± 0.70
60 nm	< DL	0.33 ± 0.05	1.4 ± 0.36	2.95 ± 0.21
100 nm	< DL	0.45 ± 0.06	1.35 ± 0.29	3.58 ± 0.91

between all the exposure concentrations with the highest oxygen consumption at the nAg concentration of 5 $\mu\text{g L}^{-1}$. For the nAg 20 nm conditions, exposure at 2.5 and 5 $\mu\text{g L}^{-1}$ induced a significantly higher oxygen consumption compared to the control. For the nAg 40 conditions, the gammarid oxygen consumption was significantly higher after exposure at 2.5 and 5 $\mu\text{g L}^{-1}$ than after exposure to the control.

3.3.4. Relationship between nAg gammarid concentrations and oxygen consumption

The linear model built between the data obtained for the silver concentrations in *G. roeseli* and the oxygen consumption was significant ($F=33.88$, df=1, 14, $P<0.001$; Fig. 4) and shows a strong effect of nAg concentration measured in gammarids on individual oxygen consumption.

4. Discussion

4.1. Silver characterization and concentrations

The experimental procedures chosen for the present study, and in particular the very low concentrations of nAg tested, did not allow the determination of the particle size distribution in the experimental media due to the too high detection limit of the Nanosight particle tracker (10^8 particles mL^{-1}). Even so, the tracking analysis was performed at higher concentration (100 $\mu\text{g L}^{-1}$). Although this is not realistic from an environmental point of view, it allowed a better understanding of the behaviour of nanoparticles in this particular medium. The nAg hydrodynamic diameters measured in the complex matrix of La Meholle water were larger than pristine nAg showing a probable complexation and agglomeration of the nAg linked to the physicochemical properties of the water. Gao et al. (2009) showed that the water chemistry (e.g: dissolved organic carbon, ionic strength and pH) might affect the nanoparticle dispersion and size distribution. For example, pristine nAg of 20 to 30 nm displayed diameters of approximately 100 nm when placed in deionized water and of approximately 400 nm in the Suwannee river water. Natural water contains natural organic matter like fulvic and humic acids that are known to play a key role in nanoparticle sorption and could mitigate their bioavailability (Bernhardt et al., 2010; Maurer-Jones et al., 2013). Regarding our results and those of previously published works, even if the measurements were not done at the same exposure concentrations as we used for gammarids, we could nevertheless assume that the effects observed on gammarids might be due to complexed or aggregated nanoparticles. This statement is supported by the work of Auffan et al. (2020) that showed an accumulation of nAg in surficial sediments of mesocosms attributed to their aggregation and settlement.

Silver nanoparticles may release ionic silver by oxidative dissolution (Liu and Hurt, 2010) and there is a general agreement that toxicity is partly due to the slow release of ionic silver from nAg (Behra et al., 2013; Kittler et al., 2010). The dissolution rate depends on the nanoparticle itself (size, coating, surface functionalization), but also on the water chemistry (temperature, pH, presence of Ag^+). In the present study, no dissolution was detected for the 5 nAg sizes tested in the stock solutions and in each microcosm after 72h. As our experiments were conducted by adding nanoparticles in natural river water, it is suggested that physicochemical conditions did not favour nAg dissolution: low temperature (12°C), natural river pH (8.1) and the presence of natural organic matter may explain the absence of dissolution process (Liu and Hurt, 2010).

Determination of Ag concentrations in *G. roeseli* after 72 h showed a concentration-dependent increase that was significant only for the exposure to the smallest nAg size (10 nm). In the present study we did not investigate whether the presence of silver results from bioaccumulation and whether silver is in nanoparticulate form in gammarids. Several studies have already documented the uptake and accumulation of Ag/nAg in aquatic organisms such as crustacea, gastropod or fish (Auffan et al., 2020; Croteau et al., 2011; Farkas et al.,

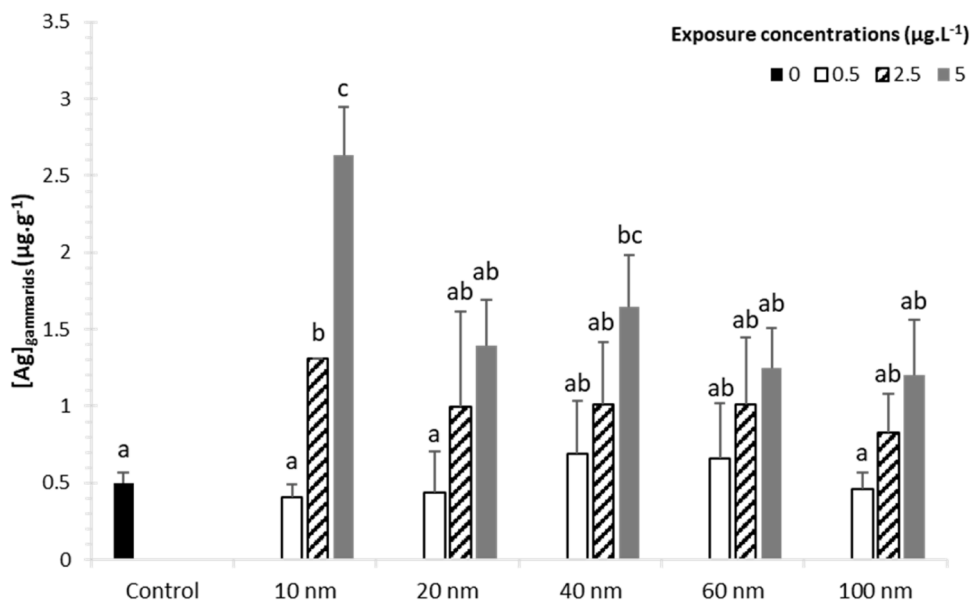


Fig. 1. Mean (+ S.D.) silver concentration in gammarids exposed to 4 concentrations (0, 0.5, 2.5 and 5 $\mu\text{g}\cdot\text{L}^{-1}$) of 5 nAg sizes (10, 20, 40, 60 and 100 nm) after 72 h. Groups with the same letters are not significantly different (one-way ANOVA; Tukey HSD; n=4).

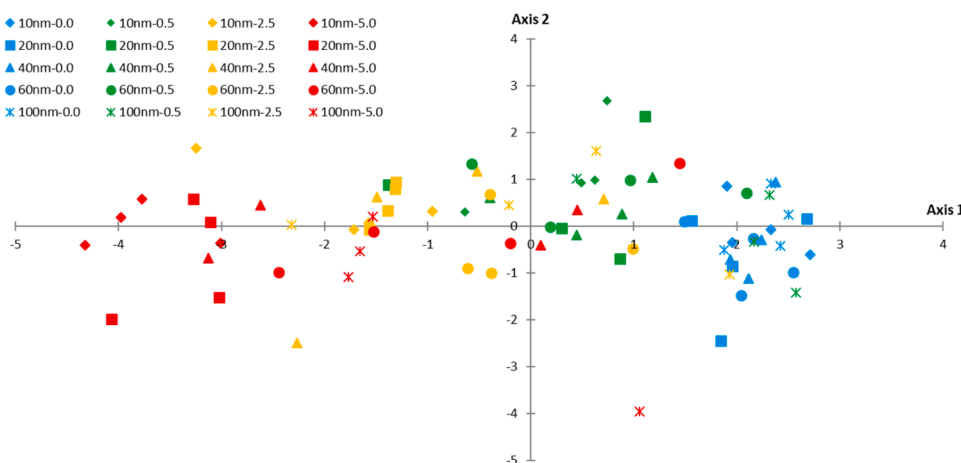


Fig. 2. Discriminant analysis coupling 5 energetic biomarker responses (oxygen consumption, ventilation, ETS activity and concentrations of triglyceride and protein) measured on the same individual, for gammarids exposed to 4 concentrations (0, 0.5, 2.5 and 5 $\mu\text{g}\cdot\text{L}^{-1}$) of 5 nAg sizes (10, 20, 40, 60 and 100 nm) after 72 h (n=4; Wilks p-value=5.07 10^{-6} ; error rate= 0.725).

2011; Mehennaoui et al., 2021) and shown that silver tissue contents was depending of the particle size (Gaiser et al., 2012). Auffan et al. (2009) found size-dependent interfacial properties and reactivity of nanoparticles which enhanced adsorption for nanoparticles of 30 nm or less and Morones et al. (2005) observed direct interaction of individual nAg with the membrane of Gram-negative bacteria preferentially for nAg of 1 to 10 nm in diameter. They also observed small nAg inside the bacteria cells, suggesting membrane interaction is needed before the uptake. After 48 h of zebrafish embryos exposure to 25 $\mu\text{g}\cdot\text{mL}^{-1}$ of small nAg (BSA coating) of 5-20 nm, Asharani et al. (2008) observed on TEM images the presence of nAg inside the cells preferentially accumulated in the nucleus. By using a NanoSIMS approach, Georgantzopoulou et al. (2013) observed nAg in the gut lumen of *D. magna* after a 48 h exposure of 0.1 $\text{mg}\cdot\text{L}^{-1}$ of 20 and 200 nm. However, and in contrast to the previously cited works, only the largest ones (200 nm) have passed the peritrophic membrane and have slightly entered into intercellular spaces within the epithelial layer. Together these results highlighted that exposure to nAg could induce accumulation of Ag in the nanoparticulate form in tissues or cells. In our study and in most of the works

reported in the literature, small nAg seem to be the most adsorbed and/or absorbed and then could be responsible for toxicity at the organism level. Indeed, the size-dependent crystalline structure of nanoparticles can influence their reactivity suggesting possible size dependence in toxicity (Auffan et al., 2009).

4.2. Biological endpoints

4.2.1. Survival

Exposure to low concentrations of nAg did not affect the survival of *G. roeseli* during the 72 h exposure period for the 5 nAg sizes studied, as already shown during experiments on gammarids exposed to similar environmentally realistic concentrations (Andrei et al., 2016; Mehennaoui et al., 2016, 2021).

4.2.2. Respiration and energetic responses

The overall result of the discriminant analysis performed with all the biological responses give a regular repartition of experimental groups along a gradient ranging from exposure to low concentrations very close

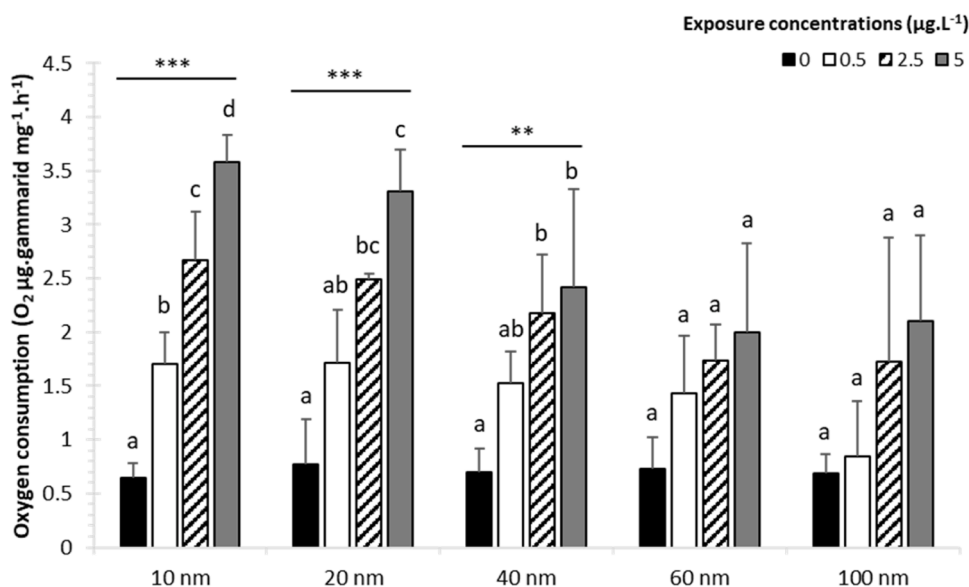


Fig. 3. Mean (+ S.D.) oxygen consumption of gammarids exposed to 4 concentrations (0, 0.5, 2.5 and 5 µg L⁻¹) of 5 nAg sizes (10, 20, 40, 60 and 100 nm) after 72 h. For each nAg size, asterisks represent the one-way ANOVA p-value results of the concentration effects and the groups with the same letters are not significantly different (Tukey HSD; n=4).

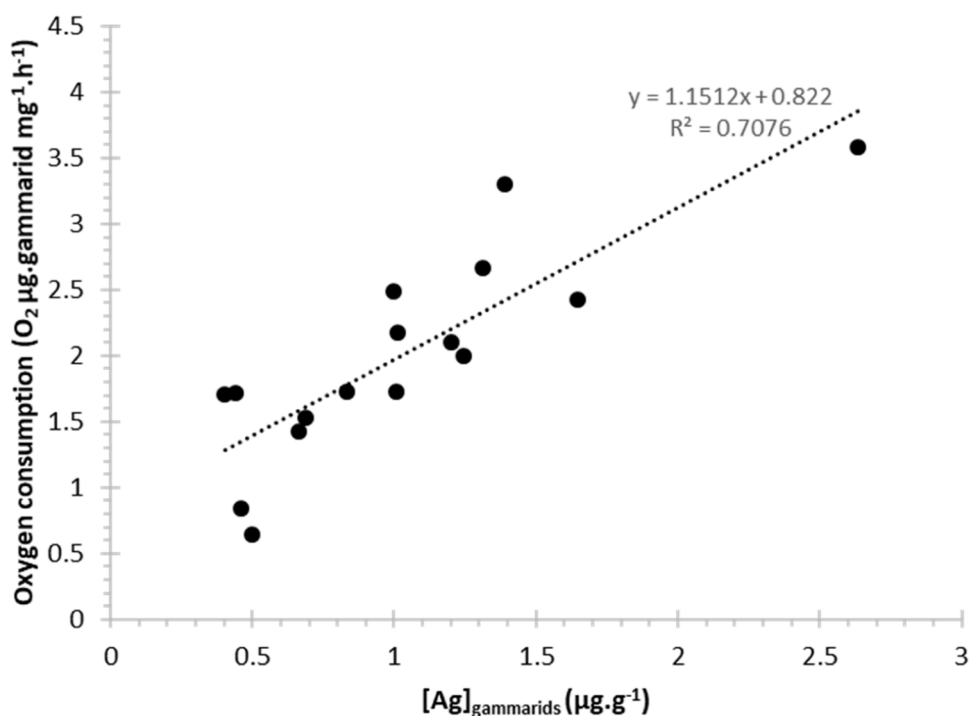


Fig. 4. Linear model built between the data obtained for the silver concentrations in gammarids and their oxygen consumption.

to the control conditions, to exposure to the highest ones. This repartition suggests that nAg biological effects were dependent on the concentrations. However, an effect of the nAg size was not clearly evidenced.

Detailing the results of the discriminant analysis enables to show that the repartition of experimental groups was driven by the oxygen consumption parameter. In our study, oxygen consumption was increased in exposed gammarids. This increase was highly significant for exposures to the smallest nAg (10 and 20 nm and in a lesser extent 40 nm), whereas it was not significant for the largest ones (60 and 100 nm). It is now well accepted that small nAg induce higher toxicity than large ones (Auffan

et al., 2009; Bar-Ilan et al., 2009; Hoheisel et al., 2012; Morones et al., 2005), what is mainly explained by a high generation of ROS by small nanoparticles (Auffan et al., 2009; Carlson et al., 2008; Choi and Hu, 2008).

Whereas the oxygen consumption by gammarids was increased in our experiments, other studies showed a decrease in other gammarids (Simcic and Brancelj, 2006; Sutcliffe, 1984) exposed to acute concentrations of metal such as copper mercury and silver (Jones, 1941). Concerning the effect of nAg exposure on oxygen consumption, studies in fish failed to demonstrate any modification (Bilberg et al., 2010; Cowart et al., 2011). For crustaceans, Ketpadung and Tangkrock-Olan

(2006) measured an increase of the respiration rate in marine blue crabs (*Portunus pelagicus*) exposed to increasing copper concentration. This increased oxygen uptake was related to the establishment of an escape response (need to increase energy supply and then ATP production in mitochondria, what requires O₂ as a final electron acceptor) or to the establishment of an internal hypoxia requiring a compensation by an increased uptake (Ketpadung and Tangkrock-Olan, 2006). Whether the increase in oxygen consumption we measured in gammarids can be the result of an escape behavior cannot be concluded from our results, but previous works reported rather a decrease of the locomotor activity after a nAg exposure (Andreï et al., 2016; Mehennaoui et al., 2016, 2021), invalidating this hypothesis for gammarids. In contrast, the establishment of an internal hypoxia may be an explanation for our results. Indeed as explained by Sutcliffe (1984), a strong difference between two partial pressures of oxygen (PO₂), for example between high PO₂ in water and low PO₂ in gammarids, induced a high oxygen diffusion rate from water to gammarids. If nAg provoked an internal hypoxia in gammarids exposed to the highest concentrations, replacing individuals in micro-respiration chambers where oxygen was at saturation may have accelerated the diffusion rate towards the gills cells, generating the high oxygen consumption we reported. One of the most studied toxicity paradigm for nAg is the generation of reactive oxygen species (ROS, Carlson et al., 2008; Choi et al., 2010; Lapresta-Fernandez et al., 2012; Xia et al., 2006) and Eom et al. (2013) demonstrated that ROS induction following nAg exposure in *Caenorhabditis elegans* may have induced the hypoxia inducible factor-1 (HIF-1), a protein acting as a transcription factor regulating physiological pathways responsible for improvement of O₂ transport. This findings support the hypothesis that hypoxia may have resulted from nAg exposure in our study.

Whereas oxygen consumption was clearly increased in our study, ventilation activity remained unchanged. For gammarids, ventilation corresponds to the beating of pleopods in front of gills, then favoring O₂ diffusion towards gills cells. It is surprising that the assumed hypoxia in gammarids was not reflected in an increased ventilation activity. However, ventilation activity was not impaired in *G. fossarum* either following nAg exposure to the same nAg concentration (Mehennaoui et al., 2021) suggesting that ventilation may not be indicative of stress in such kinds of experiments.

Likewise, ETS activity and energetic reserves remained unchanged during the exposure of gammarids to nAg, as if the uptake of oxygen was not (yet) reflecting an increased energy need. However, energetic biomarkers are known as good tools to assess the effects of a toxic stress on the organism energy budget (De Coen and Janssen, 1997; Verslycke et al., 2004). The cellular energy allocation (CEA) can be studied in organisms by measuring both energy available (Ea) namely protein, lipid and carbohydrate contents, and energy consumed (Ec) through the ETS activity allows an evaluation of an oxygen consumed rate (De Coen and Janssen, 1997; Gomes et al., 2015; Olsen et al., 2008). After pesticide exposure (0.072 and 0.100 µg L⁻¹ of chlorpyrifos), the estuarine shrimp *Neomysis integer* has been affected for both Ea and Ec with more pronounced effects for the protein content and the ETS activity (Verslycke et al., 2004). Meanwhile, the exposure of *N. integer* to the pesticide revealed a significant increase in respiration rate at increasing concentration, as observed in our study.

Further investigations are needed to confirm these results and better understand both the modification of oxygen budget in the gammarids under nAg exposure and the putative impacts on the energy metabolism. In this aim, considering the dietary route of exposure in the context of trophic transfer of nanoparticles and in the context of ecosystems processes is a relevant lead to explore (Andreï et al., 2016; Baudrimont et al., 2018; Bundschuh et al., 2016).

5. Conclusion

The present study showed that for an environmentally realistic scenario of exposure (low nAg concentrations in a natural river water), silver was recovered in the water and in *G. roeseli* after 72 h of nAg

exposure. We showed that nAg affected ecophysiological responses in *G. roeseli* and the main impact reported was an increase oxygen consumption related to the silver concentrations and to the nanoparticle sizes. Increased oxygen uptake may have been related to the establishment of internal hypoxia, but further experiments are needed to determine the mechanisms underlying these findings. Nevertheless, effects observed from the very low exposure concentration of 0.5 µg L⁻¹ for the small nAg size showed a new insight of potential environmental impact associated with the release of nAg in natural ecosystem.

CRedit authorship contribution statement

Jennifer Andreï: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **François Guérol:** Conceptualization, Validation, Writing – original draft, Supervision. **Jonathan Bouquerel:** Methodology, Investigation. **Simon Devin:** Formal analysis, Writing – review & editing. **Kahina Mehennaoui:** Methodology, Investigation, Writing – review & editing. **Sebastien Cambier:** Validation, Writing – review & editing, Supervision. **Arno C. Gutleb:** Writing – review & editing, Project administration, Funding acquisition. **Laure Giambérini:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sandrine Pain-Devin:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgements

Financial supports were provided by the Agence Nationale de la Recherche (ANR-10-NANO-0006/MESONNET project), the Laboratory of Excellence Resources 21 "Strategic metals in the 21st century" (ANR-10-LABX-21-01) and the CPER Lorraine-ZAM (Contrat Projet Etat Région Lorraine, Zone Atelier Moselle). The UE COST Action ES1205 ENTER (Engineered nanomaterials from wastewater treatment & stormwater to rivers) and the iCEINT International Consortium for the Environmental Implications of NanoTechnology are acknowledged for providing financial support to J. Andreï for attending meetings and training schools. The contribution of Kahina Mehennaoui was in part made possible within NanoGAM (AFR-PhD-9229040, Fonds National de la Recherche Luxembourg). The contributions of Sebastien Cambier and Arno C. Gutleb were partly made possible within NANION (FNR/12/SR/4009651-Fonds National de la Recherche Luxembourg). The authors wish to thank Philippe Rousselle for the AAS analyses and Carla Frongia for her help during her bibliographic internship.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106421.

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