



Original Article

Chronic Effects of Silver Nanoparticles on Micro-Crustacean *Daphnia lumholtzi*

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Abstract: This study aimed to enhance our insight on the potential toxicological effects of silver nanoparticles (AgNPs) into the aquatic environment. To investigate the chronic toxicity of nanoparticles, freshwater micro-crustacean *Daphnia lumholtzi* was exposed to different concentrations of 0.2, 0.5 µg/l AgNPs, and control, for 21 days. Toxicological endpoints at different growing stages such as the maturation and reproduction were recorded. The reproduction rate of *D. lumholtzi* exposed to both AgNPs concentrations (0.2 and 0.5 µg/l) was significantly lower than that of control. In turn, the maturation exposed to both AgNPs concentrations was not significantly different from the control treatment. This result indicates that AgNPs (with a concentration lower than 0.5 µg/l) did not have an adverse effect on the maturation of *D. lumholtzi*, but AgNPs with a concentration higher than 0.2 caused a toxic effect on the reproduction rate of *D. lumholtzi* during 21 days of the exposure period. In conclusion, the present results showed that AgNPs have toxic effects on *D. lumholtzi* and it has the potential to use as good freshwater aquatic zooplankton for assessment on the toxicity of nanomaterials in tropics. The future study should pay more attention to the effect of AgNPs on survival, growth rate, and multiple generations of daphnids to better understand the effects of nanoparticles in general and AgNPs in particular.

Keywords: Bootstrap method, chronic test, *Daphnia lumholtzi*, ecological toxicology, silver nanoparticles (AgNPs).

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1. Introduction

In recent decades, the developments of nanotechnology are steadily increasing as nanoparticles have been widely used in different industrial sectors and areas of society [1]. Nanoparticles, defined as particles with at least one dimension in the range of 1–100 nm [2], it is expected that large amounts of nanoparticles release to the environment. It was evaluated that around 0.4–7.0% of over 260,000–309,000 metric tons of nanoparticles produced globally in 2010 was discharged into aquatic environments [3]. Owing to their antimicrobial, catalytic effects, and plasmonic properties, silver nanoparticles (AgNPs) are already in use in numerous consumer and medical applications [4]. AgNPs have been widely using in numerous consumer products including textiles, personal care products, food storage containers, laundry additives, home appliances, paints, and even food supplements [5]. Therefore, an increased likelihood of AgNPs released in the aquatic environment if waste is not properly disposed and possibly exert toxic effects on aquatic organisms [2]. The list of literature has been published on AgNPs toxicity to a variety of organisms, including aquatic vertebrates, invertebrates, plants, algae, fungi, and human cells [6].

Micro-crustaceans are one of the most diverse and important groups of zooplankton. They are an important component in the freshwater food web as a trophic link between primary production and other consumers [7]. Furthermore, micro-crustaceans have been shown to be especially sensitive to engineered nanoparticles when compared to other traditional aquatic test species [8]. The aquatic crustacean *Daphnia magna* has been recognized with the first choice in ecological toxicology tests on nanoparticles [9]. In addition, the potential toxicity effects of AgNPs on *D. similis* [10], and

D. magna [11] have been reported. To the best of our knowledge, studies reporting the toxicity effects of AgNPs on *D. lumholtzi* are still scarce. In Vietnam, studies of AgNPs and its effects on aquatic organisms have until recently referred only to tropical freshwater and marine microalgae. AgNPs have resulted in a change in cell diameter, reduction in chlorophyll-a content, and enhancement of the total lipid production in the tested microalgae [12]. Overall, basic information regarding the AgNPs size, concentration, distribution, and its toxicity on aquatic organisms are unknown in Vietnam.

The aim of the present study was to evaluate the toxicity of AgNPs on aquatic crustacean (*D. lumholtzi*). The chronic toxicity of different concentrations of AgNPs on *D. lumholtzi* was assessed during 21 days of exposure. To investigate the growth and response induced by silver nanostructures in *D. lumholtzi*, the maturation and reproduction were determined.

2. Materials and methods

2.1. Preparation of silver nanoparticles

The silver nanoparticle was prepared by the chemical reduction of silver nitrate in aqueous solutions according to the methods of Becaro et al. (2015) [13]. Briefly, polyvinyl alcohol (PVA), a stabilizing agent was used to react with silver nitrate (AgNO_3) in Milli-Q water. The solution was then reduced with sodium borohydride (NaBH_4). All reagents were obtained from Sigma-Aldrich. The TEM image, UV-Vis absorbance spectrum and particle size distribution of silver nanoparticles were shown in Figure 1. The TEM measurements of the primary particle size of individual particles gave a diameter of 9.8 ± 0.8 nm measured on > 60 particles. This AgNPs was kept in dark at $4.0 \pm 1^\circ\text{C}$ and used within 6 months.

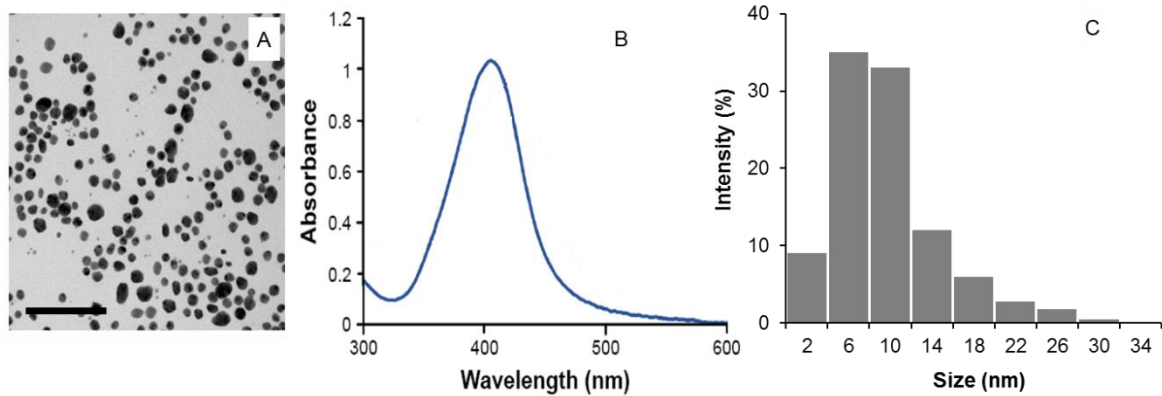


Figure 1. TEM image (A), UV-Vis absorbance spectrum (B) and particle size distribution (C) of silver nanoparticles. Scale bar: 100 nm.

2.2. Test micro-crustacean

The micro-crustacean *D. lumholtzi* (Figure 2) was isolated from a shrimp pond in Bac Ninh Province, Vietnam. This *Daphnia* was characterized by the long helmet and tail spines. The helmet is large and the tail spine is normally as long as the body length. Other distinct characteristics are the fornices that extend to a sharp point instead of being rounded, and the ventral carapace margin has approximately 10 prominent spines [14]. The life-span of *D. lumholtzi* may depend on factors such as temperature and the abundance of predators and food. In typical conditions, the life cycle is from 3–4 months. *D. lumholtzi* reproduces asexually. They produce a brood of diploid eggs. Under typical conditions, these eggs hatch after a day and remain in the female's brood pouch for around three days. They are then released into the water, and pass through a further 4–6 instars over 5–7 days before reaching an age where they are able to reproduce [15]. The micro-crustacean was maintained in 1-L beaker filled with COMBO medium [13] at $27 \pm 1^\circ\text{C}$ with a photoperiod of 12 h:12 h light:dark cycle at light intensity of $50 \mu\text{mol photons/m}^2/\text{s}$. The *Daphnia* was fed daily with a mixture (1:1 w/w) of yeast purchased from Bach Khoa Chemical Company and *Chlorella* sp. obtained from Research Institute for Aquaculture No. 2.



Figure 2. *Daphnia lumholtzi*. Scale bar: 10 μm .

2.3. Chronic tests

Chronic tests were conducted according to Clescerl et al. (2005) with minor modifications [16]. Chronic tests were performed at the same condition mentioned above. Briefly, 15 neonates (per treatment) of *D. lumholtzi* less than 24 h-age were individually incubated in 50-mL beakers containing 20 mL control solution (COMBO) or COMBO with AgNP solution at two concentrations of 0.2 and 0.5 $\mu\text{g/L}$. Test solutions were renewed every two days. The *Daphnia* was fed daily with a mixture of *Chlorella* sp. (approximately 2×10^5 cells/ml) and yeast. The mortality, maturation of the test animals and number of live offspring were recorded daily. The chronic tests lasted for 3 weeks.

2.4. Data analysis

The significant differences in *Daphnia*'s maturation and reproduction from control and AgNPs exposures were tested by the parametric test (t-test) with assumptions of homogeneity tested by the Shapiro-Wilk normality test. All analyses were performed in R [17]. In case the homogeneity of variances was not fulfilled (even not after log transformation of the data), the bootstrap method (non-parametric test) was applied with 1,000 replications, using the boot package in R [18]. The studentized 95% confidence intervals of the bootstrapped parameters were compared.

3. Results and discussion

The influence of AgNPs on the maturation of *D. lumholtzi* was showed in Figure 3A. *D. lumholtzi* in control reached their maturation after 7 days old. Furthermore, the maturity age of *D. lumholtzi* exposed to 0.2 $\mu\text{g/l}$ AgNPs was around 8 days. Exposed to AgNPs at a concentration of 0.5 $\mu\text{g/l}$, the maturation of *D. lumholtzi* was lowest than those observed in control and 0.2 $\mu\text{g/l}$, reached 6 days. As seen in Figure 3B, the clutch size of a mother *D. lumholtzi* was around 4 offsprings in control treatment. However, the clutch size of *D. lumholtzi* was decreased in the 0.2 and 0.5 $\mu\text{g/l}$ AgNPs treatments (2 and 3 offsprings, respectively).

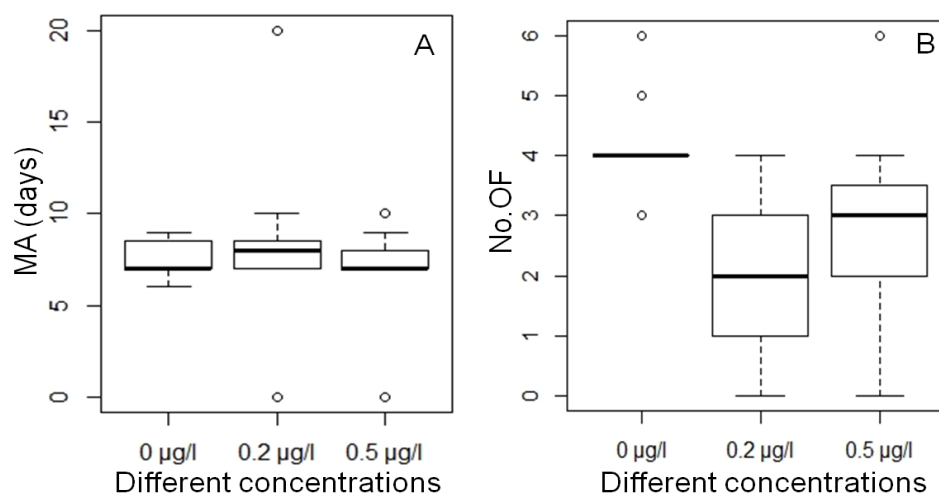


Figure 3. *Daphnia*'s boxplots data for (A) maturity ages (MA-Days), and (B) number of offspring per female (No.OF) from control (0 $\mu\text{g/l}$ AgNPs) and AgNPs exposures (0.2 and 0.5 $\mu\text{g/l}$) (n=15). Thick black lines represent the median, empty circles represent the outlier value, the lower box indicates the first quartile and the upper box indicates the third quartile. The upper line of the boxes shows the maximum value and lower line shows the minimum value.

Many of the statistical procedures including analysis of variance (ANOVA) and t-tests, namely parametric tests, are based on the assumption that the data follow a normal distribution [19]. The main tests for the assessment of normality are of Shapiro - Wilk normality test [20]. In Figure 4, both frequency distributions and Shapiro-Wilk plots show that number of offspring per female in 0.2 $\mu\text{g/l}$, 0.5

$\mu\text{g/l}$ treatment follow a normal distribution while maturity age in control, 0.2 $\mu\text{g/l}$, 0.5 $\mu\text{g/l}$; a number of offspring per female in control do not. It is clear that for number of offspring per female in 0.2 $\mu\text{g/l}$, 0.5 $\mu\text{g/l}$ have a p-value greater than 0.05, which indicates a normal distribution of data, while for others data are not normally distributed as both p values are less than 0.05.

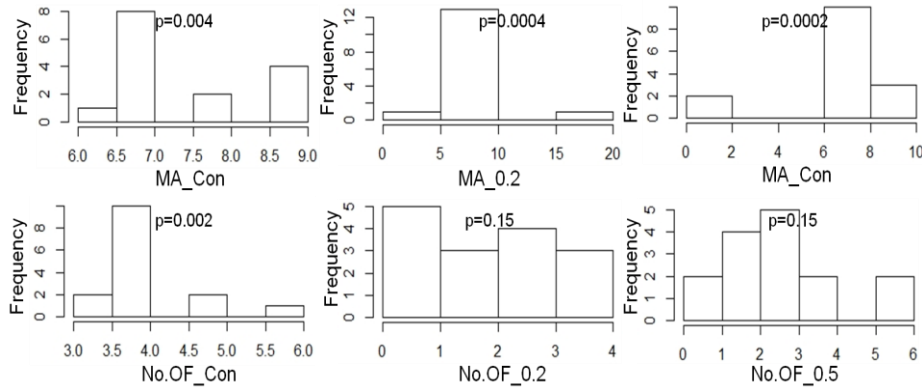


Figure 4. Results of Shapiro-Wilk normality test (MA_Con/0.2/0.5: Maturity age in control, 0.2 µg/l, 0.5 µg/l; No. OF_Con/0.2/0.5: Number of offspring per female in control, 0.2 µg/l, 0.5 µg/l).

Comparing No.OF in 0.2 µg/l and control treatment, the results of bootstrap showed that the 2.5th percentile of the bootstrap distribution was at -2.73 offspring and the 97.5th percentile was at -1.13 offspring. The combination of these results to provide a 95% confidence for mean No.OF/0.2 - mean No.OF/Con that was between -2.73 and -1.13. We could interpret this as with any confidence interval, that was 95% confident that the difference in the true means was between -2.73 and -1.13 offspring. A similar result was found in comparing No.OF in 0.5 µg/l and control. However, the mean MA/0.2 - mean

MA/Con that was between -1.27 and 2.73 days. Therefore, the mean of MA in 0.2 µg/l could be higher or lower than the mean of MA in control treatment (with a 95% confidence interval). A similar result was found in comparing MA in 0.5 µg/l and control. To summarize, the results of bootstrap analysis (with 1,000 replications) confirmed that No.OF in 0.2 and 0.5 µg/l were significantly lower than the control treatment. By contrast, MA in 0.2 and 0.5 µg/l were not significantly with control treatment (Figure 5 and Table 1).

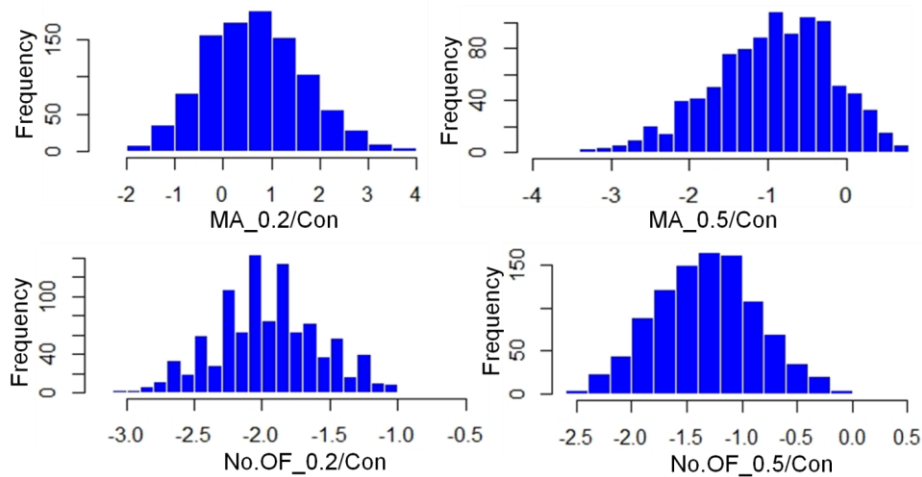


Figure 5. Histogram of bootstrap distribution with 95% bootstrap confidence intervals (MA_0.2/Con: Comparing maturity age in 0.2 µg/l and control treatment, MA_0.5/Con: Maturity age in 0.5 µg/l and control, No.OF_0.2/Con: number of offspring per female in 0.2 µg/l and control, No.OF_0.5/Con: number of offspring per female in 0.5 µg/l and control).

Table 1. Bootstrap distribution with 95% bootstrap confidence intervals

Comparing paired	Confidence intervals		
	2.5%	50%	97.5%
MA_0.2/Con	-1.27	0.60	2.73
MA_0.5/Con	-2.53	-0.80	0.47
No.OF_0.2/Con	-2.73	-1.93	-1.13
No.OF_0.5/Con	-2.13	-1.27	-0.40

Toxic effects of AgNPs on aquatic organisms have often examined using temperate *D. magna* under laboratory conditions. Nevertheless, information on both acute and chronic toxic effects of AgNPs to crustaceans, especially to those originated from tropical regions, has not been adequately investigated. The present study is one of the first study reported for the first time the chronic toxicity of AgNPs to tropical *D. lumholtzi* neonates. The present study showed that AgNPs (with a concentration lower than 0.5 $\mu\text{g/l}$) did not have an adverse effect on the maturation of *D. lumholtzi*, but AgNPs with a concentration higher than 0.2 $\mu\text{g/l}$ caused a toxic effect on the reproduction rate of *D. lumholtzi* during 21 days of the exposure period. In several studies, *Daphnia* was exposed to a higher concentration of AgNPs resulted in reducing growth and reproduction in a dose-response manner. Decreased cumulative offspring was also reported in previous studies by Zhao and Wang (2011) [21] and Blinova et al. (2013) [22] at AgNP exposures of 50 and 100 $\mu\text{g/L}$, respectively. As described previously, the authors of a study on chronic (21 d) effects of nanosilver on *D. magna* hypothesized that negative effects on growth and reproduction resulted from a reduced food intake due to the accumulation of particles in the digestive tract of the daphnids [21].

Reduced reproductive output is bound to induce population sustainability or growth. *Daphnia* communities play an important role in the food web [7] and a subtle change in the quantity and quality of *Daphnia* communities are certain to effects other populations of aquatic

organisms, resulting in major environmental impacts [23]. the alteration of *Daphnia* population might have serious consequences on the overall functioning of the aquatic ecosystem. Furthermore, AgNPs accumulated in aquatic animals, they can enter and can strongly affect the human body through the food chain [24]. In Vietnam, although there are no estimates available to date on the influences of nanoparticles size, concentration, and distribution on its toxicity for aquatic organisms, there is an increasing trend associated with this risk due to the increasing use of nanoparticles in all areas of society.

4. Conclusion

From this evidence, it is fair to conclude that AgNPs (with a concentration lower than 0.5 $\mu\text{g/l}$) have detrimental impacts on the reproduction *D. lumholtzi*. This study suggested that it is necessary to pay attention to the effect of AgNPs on survival, growth rate, and multiple generations of daphnids in order to fully assess the effects of nanoparticles in general and AgNPs in particular. Furthermore, there is a growing need to determine the implications of the presence, concentration, distribution, and toxicity of nanoparticles in aquatic ecosystems.

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