Turk. J. Fish.& Aquat. Sci. 19(12), 1009-1016
http://doi.org/10.4194/1303-2712-v19_12_03

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Toxicity of Silver Nanoparticles to Tropical Microalgae *Scenedesmus acuminatus*, *Chaetoceros gracilis* and Crustacean *Daphnia lumholtzi*

Thanh-Luu Pham[1,*]

[1]Sustainable Management of Natural Resources and Environment Research Group, Faculty of Environment and Labour Safety, Ton Duc Thang University, Ho Chi Minh City, Vietnam

Article History
Received 13 July 2018
Accepted 21 November 2018
First Online 29 November 2018

Corresponding Author
Tel.:+842839326084
E-mail: phamthanhluu@tdtu.edu.vn

Keywords
Acute and chronic tests
Algae growth inhibition
Aquatic organisms

Abstract

Silver nanoparticles (AgNPs) have been widely used to produce consumer goods that vary in type and application. Undoubtedly the increase of production and consumption of these silver-containing products has led to the entry of silver compounds into the aquatic system. In this study, we used tropical groups of aquatic organisms including the freshwater green algae *Scenedesmus acuminatus*, the marine diatom *Chaetoceros gracilis* and the microcrustacean *Daphnia lumholtzi* as model organisms to investigate the acute and chronic toxicity of AgNPs. The test organisms were exposed following specific and standardized protocols for each species/endpoints, with modifications when necessary. The endpoints recorded were growth inhibition, survival, growth of parent animals and number of live neonates produced. The EC_{50} values of AgNP for the growth inhibition of *S. acuminatus* and *C. gracilis*were 38.5 µg/L and 24.3 µg/L, respectively. The values of median lethal concentrations (24h- and 48 h-LC_{50}) of AgNPs to *D. lumholtzi* were 69.3 and 57.6 µg/L. In the chronic exposure, tested animals were exposed to AgNP from 0.1 to 5 µg/L for 21 days. Chronic effects of AgNP on survival, growth and reproduction of the microcrustacean *D. lumholtzi* were observed in concentrations higher than 0.5 µg/L.

Introduction

Nanomaterials are increasingly being used in industrial production, as well as scientific, biological, and medical research during the past decades poses a potential threat to aquatic environment (Angel, Batley, Jarolimek, & Rogers, 2013). One of the most common nanoparticles employed are based on silver. Due to the antimicrobial activity, silver nanoparticles (AgNPs) have been widely used in food storage, household products, disinfectants, textiles, cosmetics and medical devices (Ahamed, AlSalhi, & Siddiqui, 2010). Undoubtedly the increase of production and consumption of these silver-containing products has led to the entry of silver compounds into the aquatic system (Ribeiro *et al*., 2014). Concentration of these nanoparticles has been increasing in aquatic environment and can strongly affect and damage aquatic organism (Becaro *et al*., 2015). The concentration of AgNPs from non-detectable to 5 µg/L has been reported in natural water (Angel *et al*., 2013). Also, higher concentrations, over 0.1 mg/L, have been found in surface waters (Dewez & Oukarroum, 2012). AgNPs generate different toxicity profiles because of the peculiarities of the nanostate. In fact, the nanosize and huge surface area give AgNPs the potential to interact more efficiently with biological systems and producing toxicity (Ahamed *et al*., 2010). Thus, AgNPs accumulated in surface waters may present a risk to living aquatic organisms as a result of the ecotoxicity of discrete AgNPs,

agglomerated or aggregated AgNPs, transformed AgNPs to silver sulfide (Ag_2S), or Ag ions released from the AgNPs (Pachapur *et al*., 2016). Despite the obvious interest and importance of assessing the toxicity of AgNPs in surface water, there is still lack of information on the toxicity of AgNPs, especially in tropical ecosystems, to diverse group of aquatic organisms.

Previous studies have investigated the toxic effects of AgNPs on several species of freshwater environments, such as green algae, cladocerans, and fish (Ji, Long, & Lin, 2011; Blinova *et al*., 2013; Ribeiro *et al*., 2014; Sakka, Skjolding, Mackevica, Filser, & Baun, 2016). Microcrustaceans such as *Daphnia* play an important role in aquatic ecosystems. They are filter feeder on phytoplankton and serve as food for other higher trophic levels. Due to its relatively high sensitivity to toxicants, rapid reproduction, and short lifetime, it has been used extensively to study ecotoxicity. Previous studies have demonstrated the toxic effects of AgNPs on the temperate cladoceran *D. magna* under laboratory condition. AgNP toxicity have decreased feeding rates and brood production, or caused adverse effects on survival and reproduction in *D. magna* (Ribeiro *et al*., 2014). By using radiolabelled Ag-NP to quantify of AgNPs in *D. magna*, Zhao & Wang (2010) suggested that AgNPs were more efficiently assimilated by *D. magna* and more difficult to be depurated upon dietary exposure, when compared to water only exposure.

Studies on marine and freshwater algae have included observations of inhibition of growth in the freshwater green microalgae *Pseudokirchneriella subcapitata* (Ribeiro *et al*., 2014), inhibition of photosynthesis in the freshwater chlorophyte *Chlamydomonas reinhardtii (*Navarro, Wagner, Odzak, Sigg, & Behra, 2015), *and* inhibition viability and superoxide production in the marine raphidophyte *Chattonella marina (*He, Dorantes-Aranda, & Waite, *2012).* Exposure to AgNPs has resulted in progressive depletion in algal chlorophyll content, chromosome instability and mitotic disturbance, associated with morphological malformations in filamentous green algae *Pithophora oedogonia* and *Chara vulgaris (*Dash, Singh, Chaudhary, Singh, & Dash, 2012), as well as increased reactive oxygen species (ROS) formation and lipids peroxidation in freshwater microalga *Chlorella* and marine microalga *Dunaliella (*Oukarroum, Bras, Perreault, & Popovic, 2012). Although the toxicity of AgNPs has been evaluated using cladoceran and algae, data on their toxicity on organisms, especially those originated from tropical environments remain scarce and scattered. In this study we used different tropical groups of aquatic organisms including the freshwater green algae *Scenedesmus acuminatus*, the marine diatom *Chaetoceros gracilis* and the microcrustacean *Daphnia lumholtzi* as model organisms to investigate the acute and chronic toxicity of AgNPs.

Materials and Methods

Preparation of Silver Nanoparticles

To prepare stable dispersions of nanosized silver particles, chemical reduction of silver nitrate in aqueous solutions was employed. Polyvinyl alcohol (PVA) was used as stabilizing agent with silver nitrate ($AgNO_3$) in Milli-Q water, which were then reduced in the presence of sodium borohydride ($NaBH_4$) according to the methods of Becaro *et al*. (2015). AgNP solution was stored at 4.0°C before use. The shape and size distribution of AgNP was stable under this condition (Pinto *et al*., 2010). All reagents were purchased from Sigma-Aldrich. The optical properties of AgNPs were monitored by UV-Vis spectrophotometer using 260 BioThermo at the absorption spectrum from 300 to 600 nm. AgNP shape, morphology and size distribution were evaluated by a Transmission Electron Microscope (TEM) using 200 kV accelerating voltage.

Test Organisms

The freshwater green algae *S. acuminatus* was isolated from the Nhieu Loc-Thi Nghe canal and maintained in COMBO medium. The marine diatom *C. gracilis* was isolated from the Can Gio Mangrove Biosphere Reserve, Ho Chi Minh city, Vietnam and maintained in F/2 enriched seawater medium. The tropical daphnid *D. lumholtzi* was collected from a pond in Bac Ninh Province, Vietnam, and maintained in 2-L bottle filled with COMBO-anima medium (Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998). All cultures were kept at 27±1°C with a photoperiod of 12 h: 12 h light: dark cycle at light intensity of 50 µmol photons/m^2/s. The *Daphnia* was fed daily with green alga (*Scenedesmus* sp.) cultured in COMBO medium and a mixture of yeast, cerrophyl and trout chow digestion (YCT) prepared according to the U.S. Environmental Protection Agency Method (USEPA, 2002).

Algae Growth Inhibition Test

The growth rate inhibition of *S. acuminatus* and *C. gracilis* was conducted according to the methods reported by Ribeiro *et al*. (2014). Briefly, the microalgae starting concentration of 5×10^4 cells/mL were culture in glass tubes with the design concentration of 0, 1, 10, 20, 50, 100 µg/L AgNP. Three replicates were performed per concentration. Test tubes were incubated in a light-temperature controlled chamber at 27°C for 96h with a photoperiod of 12 h:12 h light–dark. Test tubes were gently shaken one a day to re-suspend any settled cells. The cell number density was monitored every day by using a Sedgewick Rafter counting champer. The concentration that inhibits algal growth rate by 50% over 96 h (EC_{50}-

96h) was determined basing on the relative inhibition of growth rate as a function of the AgNP concentration (µg/L). The average of the specific growth rate for each period was obtained as the biomass increase after 96 h, by the following equation:

$$\mu_i - j = \frac{\ln Cj - \ln Ci}{t_j - t_i}$$

where μ_{i-j} is the average specific growth rate from time *i* to time *j*, t_i is the initial time of the exposure period, t_j is the final time of exposure, C_i is the concentration of cells at time *i* and C_j is the concentration of cells at time *j*.

Percentage inhibition of growth was calculated as:

$$\%Ir = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

where *%Ir* is the percent inhibition in average specific growth rate; μ_C is the mean value for average specific growth rate (µ) in the control group and μ_T is the average specific growth rate for the treatment replicate.

Acute Toxicity Tests

Immobilization tests were performed on *D. lumholtzi* neonates that were less than 24 h old, using the AgNP suspension concentrations from 1 to 120 µg/L according to the Protocol 202 of the Organization for the Economical Cooperation and Development (OECD, 2004). Briefly, ten neonates were placed in a glass beaker (experimental unit) containing 30 mL of test-solution. A total of 30 organisms were tested at concentrations of 1; 2; 5; 10; 20; 50; 70; 100 and 120 µg/L divided in three replicates. Immobilization was determined visually after 24 h and 48 h at each concentration. *Daphnid* was examined and photographed after 24 h and 48 h under a microscope (B100BF, Optika) equipped with a digital camera.

Chronic Tests

Chronic tests were performed at the same condition as in acute toxicity test. The AgNP concentrations in chronic tests were 0.1, 0.5, 1, 2 and 5 µg/L. Chronic tests were performed according to the Clescerl, Greenberg, and Eaton (2005) with minor modifications. Briefly, neonates (15 individuals per treatment) of *D. lumholtzi*< 24 h-age were individually incubated in 50-mL polypropylene cups containing 20 mL control solution or exposure solutions. Each treatment contained 15 replicates (n = 15). Test solutions were renewed every second day. The *Daphnia* was fed daily with a mixture of *Scenedesmus* sp. (approximately 2×10^5 cells/mL) and YTC. Mortality, maturation of the test animals and production of live offspring were recorded daily. Each mother daphnid was checked daily for numbers of neonates per clutch. Reproduction was calculated as total accumulated offspring reproduced by all mother daphnids in each treatment. Fecundity was defined as the average number of offspring in one clutch reproduced by one mother daphnid. The chronic tests lasted for 3 weeks.

Data Analyses

Percentage inhibition of growth was calculated as the method of Ribeiro *et al*. 2014. The effective concentration (EC_{50}) values for all endpoints were calculated by non-linear regression. Median lethal concentrations (LC_{50}) values with the 95% confidence interval were calculated by using Probit analyses according to the method of Stephan (1977). Kruskal-Wallis test (Sigma Plot, version 12) was applied for calculation the significant difference of the maturation, fecundity and body length of *D. lumholtzi* between control and treatments.

Results

Characterization of AgNPs

The spectrum of UV-Vis detection revealed that AgNP synthesis yielded a single and well-defined peak in the absorbance spectrum, with maximum absorbance at 400 nm. The analysis of AgNP stock suspension using TEM measurements of primary particle size of individual particles gave a diameter of 9.8±0.8 nm measured on > 60 particles (Figure 1).

Growth Inhibition Test

The EC_{50} values of AgNP for the growth inhibition of *S. acuminatus* and *C. gracilis*(after 96 h) were 38.5 µg/L and 24.3 µg/L, respectively. AgNP caused significant effects and dose-dependent increases on the growth-inhibition of *S. acuminatus* and *C. gracilis*. Significant differences from the control growth rates were detected at the concentration of 50 µg/L or higher in *S. acuminatus*, or at the concentration of 20 µg/L or higher in *C. gracilis*. AgNPs at the concentration of 100 µg/L inhibited almost 100% the growth of *S. acuminatus* whereas at 50 µg/L or higher, AgNP completely inhibited the growth of *C. gracilis* (Figure 2).

Acute and Chronic Tests

Mortality didn't occur in the control during the experimental time of acute test. The highest tested concentration of AgNP (120 µg/L) resulted in 100% mortality of *Daphnia* daphnids. AgNP showed higher toxicity to *D. lumholtzi*. The immobilization of *D. lumholtzi* neonates was recorded after 24 h and 48 h of

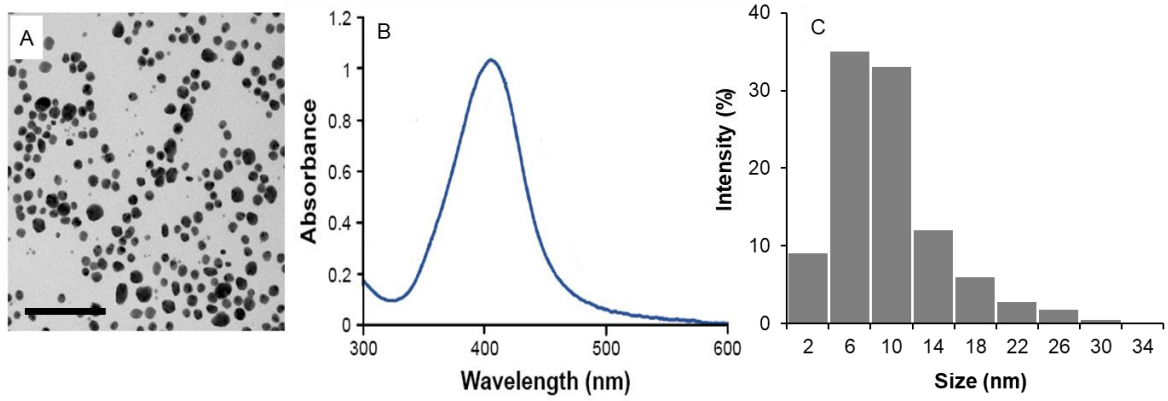

Figure 1. TEM image (A), UV-Vis absorbance spectrum (B) and particle size distribution (C) of silver nanoparticles. Scale bar: 100 nm.

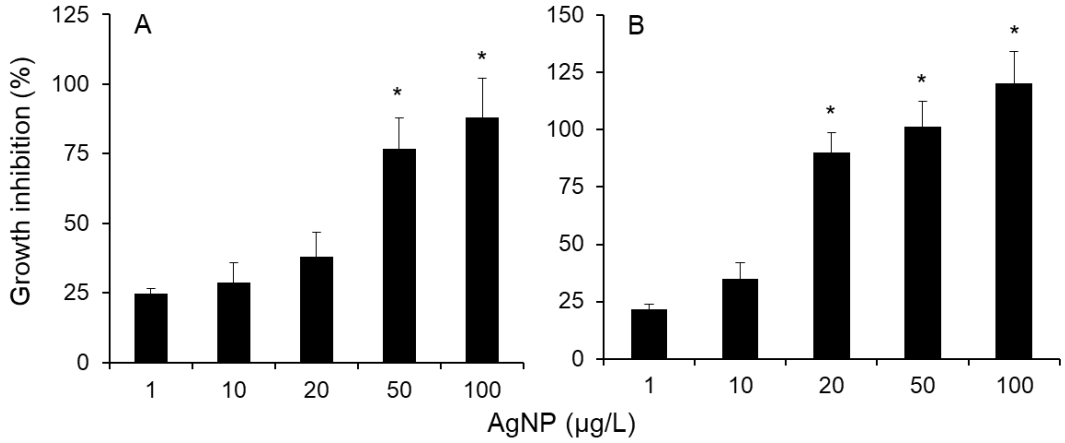

Figure 2. Growth inhibition of (A) *Scenedesmus acuminatus* and (B) *Chaetoceros gracilis* after a 96h exposure to silver nitrate and silver nanoparticles. Asterisks indicate significant differences in growth rate compared to control group. Anova, P<0.05.

exposure to AgNP. The 24 h- and 48 h-LC_{50} were 69.3 µg/L and 57.6 µg/L, respectively.

According to the OECD guideline, the control group matched the validity criterion for mortality (≤15%) in the chronic test. The chronic effects of AgNP concentrations on the survival and reproduction of *D. lumholtzi* during 21 days were shown in Figure 3. *D. lumholtzi* grew well in the control (the length of *D. lumholtzi* increased up to 2.4 mm after 21 days of incubation). While survivorship of *D. lumholtzi* decreased with increasing concentration of AgNPs in exposures during 21 days test. Significant differences in life history responses were observed for *D. lumholtzi* exposure to AgNPs. Adverse effects of AgNP on survival, growth and reproduction of the microcrustacean *D. lumholtzi* were observed in concentrations higher than 0.5 µg/L. Exposure to AgNP at concentration of 0.1, 0.5, 1, 2 and 5 µg/L caused mortality of 20%, 33%, 40%, 47% and 100%, respectively (Table 1). AgNP at the concentration of 5 µg/L induced 100% mortality on day 16 (Table 1, Figure 3).

Both growth and reproduction of parent *D. lumholtzi* were inhibited with increasing concentrations of AgNP. The time to first reproduction showed some different among treatments and control. In the control (CT) the maturity age of *D. lumholtzi* was 3.8 days (Table 1). Exposure to AgNP at the concentration of 0.1 µg/L caused no difference in maturity age of *D. lumholtzi* whereas exposure to AgNP at the concentration 0.5 µg/L or higher resulted in a significant postponement of the daphnids' maturation (Figure 4A).

During the chronic test, one adult *D. lumholtzi in the CT* produced around 29 offspring per female, and no significant different with the exposure at 0.1 µg/L. However, exposure to AgNP at concentration of 0.5 µg/L or higher resulted in significant decrease of number offspring per female. The number of neonates per female from the CT, and exposure at 0.1, 0.5, 1, 2 and 5 µg/L were 29, 28, 22, 17, 15 and 5 respectively (Figure 4B). Results of the offspring produced per living parent daphnid showed a concentration-response pattern.

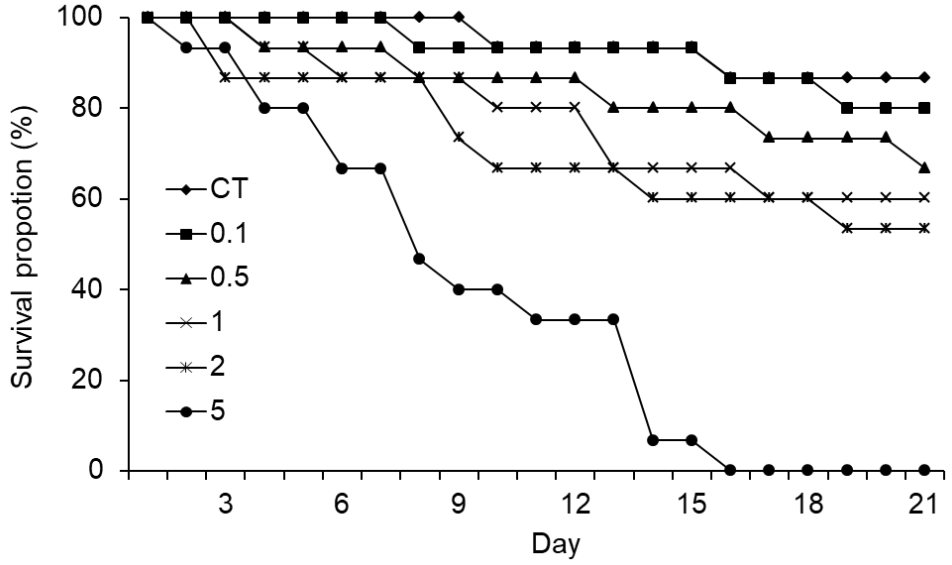

Figure 3. Effects of AgNP on survival of *D. lumholtzi*.

Table 1. Exposure concentrations and toxicity endpoints for *D. lumholtzi* after exposure to different AgNP levels for 21 days. Data were presented as mean±standard deviations.

Treatment (µg/L)	Survival (%)	Time to first brood (d)	Number of offsprings per female	Longevity (days)	Length (mm)
CT	87	3.8±0.3	29±4	21	2.4±0.04
0.1	80	4.0±0.1	28±5	21	2.3±0.05
0.5	67	4.5±0.2*	22±3*	21	2.2±0.07
1	60	4.7±0.1*	17±3**	21	2.0±0.05
2	53	5.1±0.3**	15±3**	21	2.1±0.08
5	0	5.3±0.4**	5±2***	15	1.9±0.05

Asterisks indicate significant differences compared to control group. Anova test (*, P<0.05; **, P<0.01; ***, P<0.001).

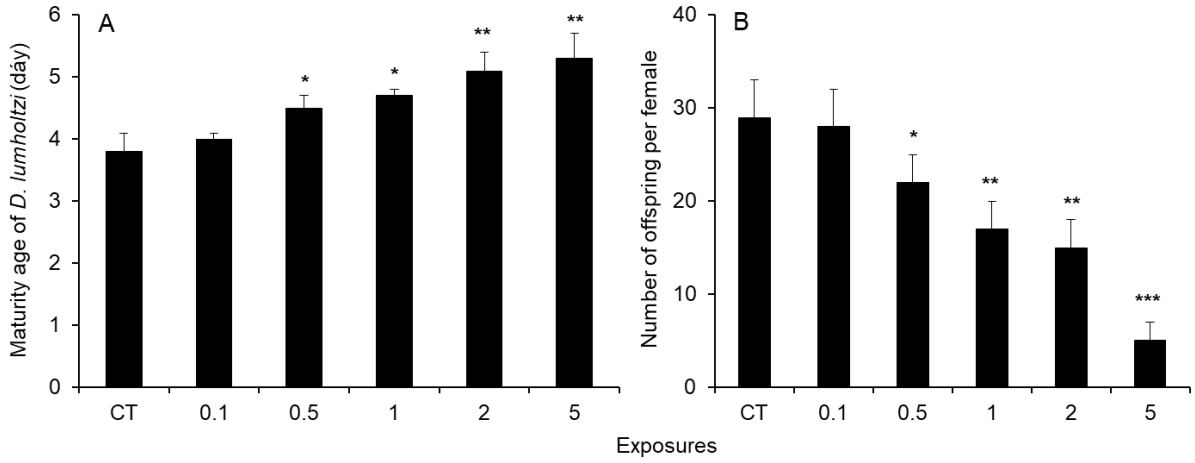

Figure 4. Maturity age (A) and number of offspring per female (B) of *Daphnia lumholtzi.* Asterisks indicate significant difference between control and exposures by Anova test (*, P<0.05; **, P<0.01; ***, P<0.001).

Discussion

Previous studies have shown that metallic NPs caused toxic effects on microalgae, which depend on the species type, exposure time, NPs concentration, and culture medium (Manzo, Miglietta, Rametta, Buono, & Di, 2013; Ribeiro *et al*., 2014; Aravantinou, Tsarpali, Dailianis, & Manariotis, 2015; Moreno-Garrido, Pérez, & Blasco, 2015; Zhang, Goswami, Xie, Zhang, & He, 2017). Few studies have focused on toxic effects of NPs on different organisms. The species type of microalgae (freshwater or marine) define the behavior of NPs and the toxic effects mechanisms since NPs dissolution depend on the aqueous matrix composition (pH, ionic strength, organic matter content, etc.) (Zhang *et al*., 2017). Manzo *et al*. (2013) observed that ZnO NPs were more toxic to the marine microalgae *Dunaliella tertiolecta* than bulk ZnO, while the adverse effects may not be strictly related to the action of zinc metal ion. Aravantinou *et al*. (2015) examined the toxic effects of ZnO NPs on two freshwater (*Chlorococcum* sp. and *Scenedesmus rubescens*) and two marine (*D. tertiolecta* and *Tetraselmis suesica*) microalgae species. The authors reported that sensitivity of algae to ZnO NPs strongly depended on the species type and the dose of NPs in exposures. Our results agree well with Aravantinou *et al*. (2015) that *Scenedesmus* showed higher viability compared to the other species. In the present study, the green algae *S*. *acuminatus* was isolated from a polluted canal in Ho Chi Minh city. Its tolerance is better than the marine *C. gracilis* who was isolated from unpolluted water environment. Previous study has showed that several strains of cyanobacteria collected from a metal contaminated wetland have metal resistance property (Sen *et al*., 2017). Probably, the *S*. *acuminatus* has developed *resistance*to environmental *pollutants.* This finding builds on previous studies and addresses a prior data gap of using difference tolerance of microalgae for identifying toxic effects of AgNPs.

Despite the continuous effort to understand the various aspects of the toxicity of AgNPs in microalgae, the cytotoxicity mechanism of AgNPs to biological organisms has remained unclear. By exploiting the unique fluorescence properties of silver nanoclusters (AgNCs) along with various other analytical/biological tools, Zhang *et al*. (2017) reported that the photosynthetic toxicity of AgNCs was largely attributed to adverse effect of particulate form of Ag^+, which resulted in the disruption of the electron transport chain of light reaction and stimulated the key enzymes (carboxylase/oxygenase) of Calvin cycle of algae cells. Another toxicity mechanism of NPs in microalgae is the formation of large number of aggregates by interaction with the cell wall or adsorb onto the external cell surface, which resulted in the impairment cell division with consequent decrease on growth rate (Aruoja, Dubourguier, Kasemets, & Kahru, 2009; Ji *et al*., 2011; Handy *et al*., 2012; Oukarroum *et al*., 2012). In *Daphnia*, the toxic effects of AgNPs may depend on the AgNPs concentration accumulated in *Daphnia* organs (Ribeiro *et al*., 2017). Once inside the animal cells, NPs may induce the production of reactive oxygen species (ROS), protein denaturation and DNA damage (Zhao & Wang, 2011). The most accepted mechanism to explain AgNPs toxicity in aquatic invertebrates such as *D. magna* is the competition of Ag^+ ion with Na^+ for the binding sites at the enzyme Na^+, K^+-ATPase, which plays an important role on the transport of Na^+ and Cl^- from water to the extracellular fluid in invertebrates (Bianchini & Wood, 2002; Mackevica, Skjolding, Gergs, Palmqvist, & Baun, 2015). This will cause inhibition of the Na^+ channels at the gill membrane and acute failure and death of the tested animal (Bianchini & Wood, 2002). In this work we aimed to investigate toxicity of AgNP to three species that represents different levels of an aquatic trophic chain. Our data suggests that the toxicity of AgNP to the all test species is dose–response dependent. The main toxicity of AgNP has been associated with the release of Ag^+ ion even though other Ag species may also available to the organisms (Ribeiro *et al*., 2017). Our results supported that higher concentration of AgNP may increase bioavailable Ag^+ in the test medium, which resulted in higher toxicity to living organisms.

Toxic effects of NPs on aquatic organisms are often investigated using temperate *D. magna* under laboratory conditions. However, information on both acute and chronic toxic effects of NPs to crustaceans, especially to those originated from tropical regions is scarce. The present study reported for the first time the acute and chronic toxicity of AgNPs to tropical *D. lumholtzi* neonates. AgNPs 48-h-LC_{50} values reported in this study were in range with 48-h-LC_{50} values for microcrustaceans *D. magna* (Mackevica *et al*., 2015) but lower than 48-h-LC_{50} values for neonate of *Daphnia similis,* or higher than 48-h-LC_{50} values for other cladocerans such as *Artemiasalina*, *Thamnocephalus platyurus* (Zhao & Wang, 2011; Becaro *et al*., 2015). These results suggested that the tropical *D. lumholtzi* may serve as a suitable surrogate for its brother *D. magna* as a model freshwater cladoceran in tropical conditions.

The present study showed that AgNPs affect survival, growth and reproduction of *D. lumholtzi*. Exposure to higher concentration of AgNPs resulted in reducing growth and reproduction in a dose-response manner. Similar to our findings for *D. magna* exposed to AgNPs at the concentration from 10 to 50 µg/L, Mackevica *et al*. (2015) reported a decrease in survival rate as well as reduce cumulative offspring per living parent, or *prolongation* the *time* to the *first brood.* Decreased cumulative offspring had *also reported in previous* studies by Zhao and Wang (2011) and Blinova *et al*. (2013) at AgNP exposures of 50 and 100 µg/L,

respectively. Reduced reproductive output may lead to induce population sustainability or growth. To the best of our knowledge, this is the first study to present chronic toxicity of AgNPs on tropical cladoceran *D. lumholtzi*. *Daphnia* communities play an important role in food web and the alteration of *Daphnia* population might have serious consequences on the overall functioning of the aquatic ecosystem.

Conclusions

The present study confirmed the adverse effects of AgNPs to different tropical aquatic organisms including alga and microcrustacean. Considering the variability of production of AgNPs as well as their routes of entry into the environment, it is important to have information on effects of AgNP at environmentally *relevant concentrations. Results of this study* suggested that there are perspectives for future research to obtain better understanding various toxic effects of AgNPs in tropical aquatic ecosystem including bioaccumulation and biotransfer. Microalgae as well as microcrustacean play an important role in the aquatic ecosystem, not only producing primary biomass as a basic nourishment for the food webs, but also contributing to the self-purification of polluted water. Therefore, a negative effect on these elements might cause serious consequences on structure and function of other aquatic communities.

Acknowledgement

I would like to thank BSc. Mymy and BSc. Giang for taking care of microalgae in laboratory. This study was partly supported by the Ton Duc Thang University undergrantnumber **"**103/2018/TĐT-HĐLV-NCV".

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