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Growth Inhibition of Aquatic Plant Caused by Silver and Titanium Oxide Nanoparticles

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Abstract

Emerging nanomaterials are of great concern to aquatic environment. The inhibitory effects of silver nanoparticles (Ag-NP) and titanium oxide nanoparticles (TiO_2-NP) on the growth of aquatic plant duckweed were evaluated. *Lemna paucicostata* was used as a test organism, and the test method was followed by the OECD test guideline 221. Ag-NP (50 nm) and TiO_2-NP (2-3 nm) inhibited the growth of Lemna in different manner and the EC50s were calculated as 13.8 and 538.5 ppm, respectively. The LOECs of Ag-NP and TiO_2-NP were calculated to be 1 and 125 ppm, respectively. The results showed that Ag-NP could cause growth inhibition of Lemna in low concentration range ($\geq$1 ppm), but TiO_2-NP appeared toxic effect to the growth of Lemna in the concentration range of $\geq$250 ppm, which was over two orders higher than the previously reported concentration of TiO_2-NP determined in the aquatic environment.

Keywords: *Lemna paucicostata*, Silver nanoparticle, Titanium oxide, Silver chloride, Aquatic plant toxicity

Introduction

Manufactured nanoparticles have received a particular attention for their positive impact in improving many consumer products, and are increasingly used for a wide range of industrial applications[1]. Nanoparticles are atomic or molecular aggregates with at least one dimension between 1 and 100 nm[2], that can drastically modify their physico-chemical properties compared to the bulk material[3]. Manufactured nanoparticles can be made from various bulk materials and they behave differently depending on both the chemical composition and on the size and/or shape of the particles[4]. There is now an extensive debate about the risks and benefits of the many manufactured nanoparticles into the environment, and in order to evaluate their potential adverse effects on the ecosystems and on human health, it has been studied with increasing attention to this topic.

It is expected that manufactured nanoparticles will be distributed into aquatic, terrestrial and atmospheric environments, where their fate and behavior are largely unknown. In the review of Navarro *et al.* (2008)[5], they described that the biotransformation of nanoparticles in contact with algae, fungi and plants, the mechanism of toxicity, the effect on organisms, and how these toxic effects might be transferred through food chains and thus affect whole ecosystems.

The potential of manufactured nanoparticles to react with biological systems has been recognized in recent years and a number of ecotoxicity studies of these emerging pollutants have appeared. Among those studies, considerable attention was paid to TiO_2 and Ag nanoparticles.

From the perspective of ecotoxicity, TiO_2 nanoparticles (TiO_2-NP) are by far the most extensively studied metal oxide nanoparticles[6,7]. One of the reasons for the large amount of toxicity data on TiO_2-NP is the adoption of this nanoparticle by a variety of industries; TiO_2-NP was the first nanoparticle made commercially available to various industrial and research activities. TiO_2 is a naturally occurring mineral that can exist in three crystalline forms, known as rutile, anatase, and brookite, and in an amorphous form[8]. Commercial production of TiO_2-NP between 2006 and 2010 was estimated as 5,000 metric tons per year, more than 10,000 metric tons per year between 2011 and 2014[9] and approximately 2.5 million metric tons by 2025[10].

TiO_2-NP has been used for a wide variety of applications, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water treatment agents and topical sunscreens[11]. Such widespread use of TiO_2-NP could lead to significant release of TiO_2-NP into the environment,

resulting to a potential for increased environmental exposure to TiO_2-NP[12].

Much knowledge already exists on the effects of TiO_2-NP on biological systems. TiO_2-NP is photoinducible, redox active and thus a generator of potential reactive oxygen species (ROS) at its surfaces. TiO_2-NP has been shown to generate ROS in the presence of UV light[13] or in its absence of UV light[14]. However, the precise mechanisms of toxicity of TiO_2-NP are largely unknown[15].

Silver nanoparticles (Ag-NP) represent another prominent nanoproduct with potential applications in medicine and hygiene. Ag-NP has antimicrobial activity resulting in their widespread use in various consumer products such as water purification, toothpaste, nursing bottles, fabrics, deodorants, filters, and humidifiers[16]. The antimicrobial properties of silver have been known for thousands of years[17], but Ag-NP has gained attention recently, because different formulations of Ag-NP with different shapes and sizes exhibit variable antimicrobial activity, which offers their possible use for medical and hygienic purposes[18].

Comparing the antimicrobial effect of silver ions and Ag-NP is an interesting field of research. Silver ions and Ag-NP have inhibitory and lethal effects on bacterial species such as *E. coli*[19], *Staphylococcus aureus*, and yeast[20]. However, Morones *et al.*[17] indicated that the mechanism of Ag-NP antimicrobial activity is different than the mechanism induced by silver ions. It was observed complete disruption of the bacterial membrane after a few minutes in contact with Ag-NP and proposed that the high efficiency of Ag-NP was due to the large surface area available for interactions, resulting in their effectiveness against bacteria[21,22]. This high efficiency occurs at nanomolar concentrations in the case of Ag-NP and in the micromolar range in the case of silver ions[23].

For these reasons, there is an emerging amount of literature on the ecotoxicity of TiO_2-NP and Ag-NP, with a majority of the studies dealing with aquatic animals, e.g., freshwater invertebrates and fish[24]. However, almost no information on the toxic effects of TiO_2-NP and Ag-NP on aquatic plants is available.

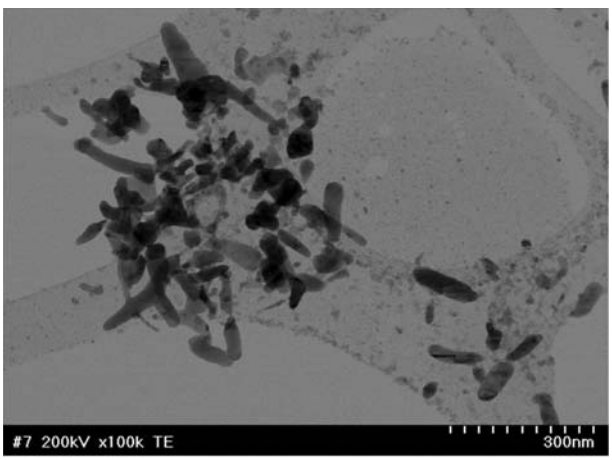
Lemna is an aquatic plant which often forms dense floating mats in eutrophic ditches and ponds[25]. These macrophytes grow fast, adapt easily to various conditions and can tolerate a wide pH range (4.5-8.3). The small size, simple structure and rapid growth make Lemna very suitable for toxicity tests[26]. It is also used in wastewater treatment to remove mineral and organic contamination and radionuclides[27].

The present study investigates the effect of Ag-NP and TiO_2-NP on *Lemna paucicostata* to assess tolerance of this aquatic plant to nanoparticle exposure. This effect is determined from the concentration that results in a 50% reduction in the growth (EC50), and the lowest observed effect concentration (LOEC).

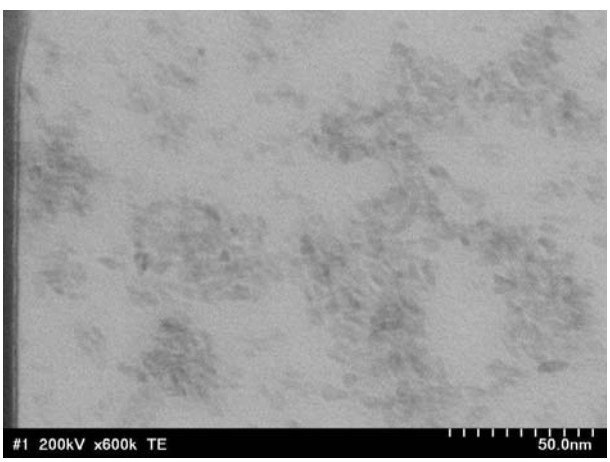
Results and Discussion

TEM Analysis of Nanoparticles

Ag-NP and TiO_2-NP used in this study were analyzed by transmission electron microscopy (TEM). The size and shape of nanoparticles were verified by TEM images. The particle size of Ag-NP was confirmed to be ca. 50 nm, as noted by manufacturer (Figure 1(a)). TiO_2-NP was verified to be 2-3 nm in particle size (Figure 1(b)), similar to the result of Lakshminarasimhan *et al.* (2007)[28]. As seen in Figure 1, both Ag-NP and TiO_2-NP showed aggregation of small primary



(a)



(b)

Figure 1. TEM analysis of nanoparticles. (a), Ag-NP; (b), TiO_2-NP.

particles into a larger secondary structure.

Growth Inhibition by Ag-NP and TiO_2-NP

Lemna was grown for 7 days on AAP media supplemented with different concentrations of Ag-NP and TiO_2-NP. The specific growth rate of Lemna was affected by Ag-NP and TiO_2-NP in a different manner. The exposure concentration versus specific growth rate curve is presented in Figure 2. It showed that exposure to TiO_2-NP resulted in relatively modest effects to Lemna than to Ag-NP.

Lemna exposed to ≥ 1 ppm Ag-NP showed decreased specific growth rate with statistical significance (Dunnett test, $p < 0.05$). When Lemna was exposed to Ag-NP ≥ 100 ppm, the growth was completely inhibited. On the other hand, TiO_2-NP in ≤ 125 ppm concentration range exerted no statistically significant effect on Lemna growth (Dunnett test, $p < 0.05$). The specific growth rate of Lemna exposed to 500 ppm of TiO_2-NP was decreased to 51% of the specific growth rate of the control.

Shaprio-Wilk's test indicated that the test data showed normal distribution ($p > 0.05$). Bartlett's tests indicated equal variance of the test data ($p = 0.92$ in Ag-NP; $p = 0.25$ in TiO_2-NP).

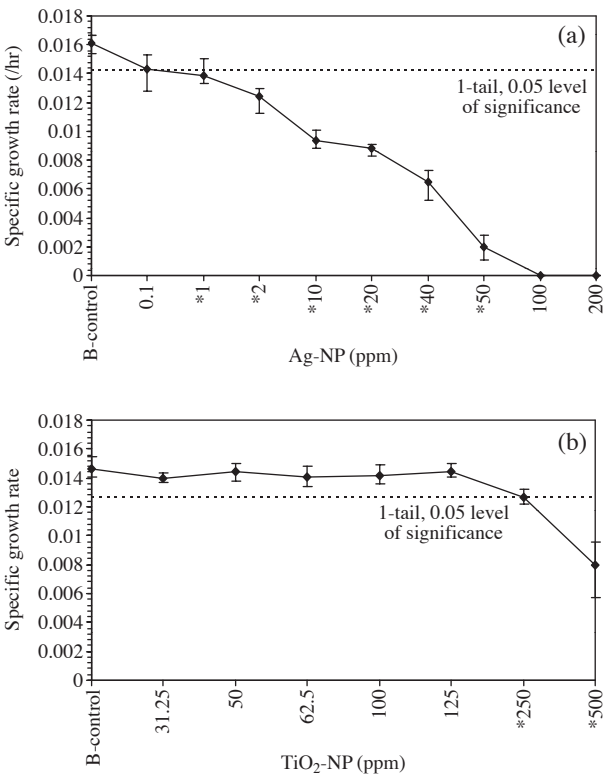


Figure 2. Growth inhibition of *L. paucicostata* caused by (a) Ag-NP, (b) TiO_2-NP.

The Ag-NP and TiO_2-NP caused other visible damages to Lemna fronds such as chlorosis. Chlorosis (a progression of green to yellow color on the frond) and frond disconnection (detachment of fronds from colonies) were also toxicity signs observed during exposure period to the nanoparticles. At the concentrations of ≥ 0.1 ppm Ag-NP, fronds were chlorotic and some fronds separated from the others. In case of TiO_2-NP, ≥ 250 ppm TiO_2-NP in the test medium caused chlorosis.

Growth Inhibition Parameters

The calculated concentration that results in a 50% reduction in the growth of Lemna (EC50) in the presence of nanoparticles was interpolated using maximum likelihood-logit method with the specific growth rate as a function of concentration. The EC50s of Ag-NP and TiO_2-NP for the specific growth rate of *L. paucicostata* were 13.8 and 538.5 ppm, respectively (Table 1). Under the 95% confidential limit, EC50 values were determined in the range of 8.4-20.4 ppm of Ag-NP, and 456.4-755.5 ppm of TiO_2-NP. The lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) were also estimated based on the Dunnett's test. The LOECs of Ag-NP and TiO_2-NP were determined as 1 and 250 ppm, respectively. The values of parameters are shown in Table 1.

Determination of Nanoparticle Concentration in Culture Media

Elemental analysis of Ag and Ti concentrations in culture media were performed using ICP-AES at Day 0 and Day 7 of the experiment (Figure 3). Unfortunately, there were serious losses of Ag and Ti in 0.45 μm filtrate of the test media. In case of Ag-NP treated media, Ag concentration by elemental analysis on Day 0 was determined to 0.029, 0.063, and 0.197 ppm, for the nominal concentration of 10, 20 and 40 ppm, respectively. This means that the only 0.3-0.5% of administrated Ag was detected in the filtrate. In case of TiO_2-NP treated test media, Ti was not detected (< 0.005 ppm) in all of the samples tested (data not shown).

As seen in Figure 1, the secondary structure of Ag-

Table 1. Parameters for growth inhibition of *L. paucicostata* exposed to nanoparticles.

Test chemical	EC50 (95% confidential limit) (ppm)	LOEC (NOEC) (ppm)
Ag-NP	13.8 (8.4-20.4)	1 (0.1)
TiO_2-NP	538.5 (456.4-755.5)	250 (125)

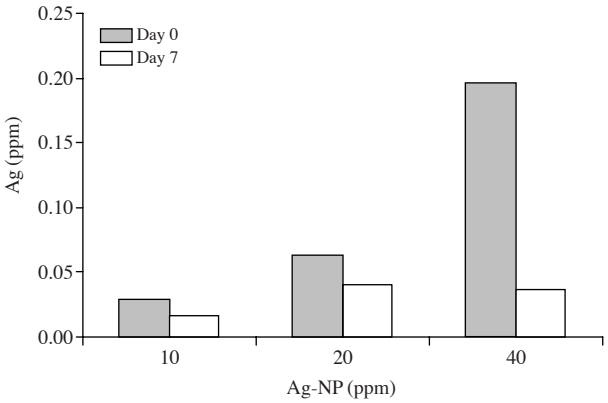


Figure 3. Elemental analysis of Ag concentration in test media.

NP and TiO_2-NP dispersed in distilled water were not exceeded the pore size (0.45 μm=450 nm) of the filter used in the sampling process for ICP-AES. However, most of particles dispersed in test media seemed to be removed during filtration process. Of course other sources for the loss of nanoparticles might exist during the experiment, e.g., adsorption of particles to the wall of the test vessels or syringe, but it might be negligible when compared to the large amount of total elemental losses.

It is estimated that once nanoparticles are introduced into culture media, which is composed of various mineral salts, they could aggregate easily and form bigger particles. Generally, colloidal particles are prevented from aggregating by electrostatic repulsion of the particle surface. But they can be coagulated (aggregated) by addition of salts, which occurs by increase of ionic strength and decrease of electrostatic repulsion. It is reported that the extent of aggregation is governed by pH, ionic strength, and the nature of the electrolytes[5].

Because precipitation of the nanoparticles was not observed in test media on Day 0, it can be hypothesized that when nanoparticles were administrated to Lemna culture media, coagulation was occurred to larger size than in distilled water. This means that the Ag-NP and TiO_2-NP toxicity test was performed in larger secondary structure than observed in TEM analysis.

Ag concentration by the elemental analysis in test media was decreased at Day 7 compared to Day 0. Figure 3 shows that Ag concentrations of 10, 20, and 40 ppm test media were remained 55%, 65%, and 19% at Day 7 respectively, compared to Day 0. The Ag concentration ratio (Day 7 to Day 0) in 40 ppm Ag-NP media was remarkably decreased, due to the similar level of Ag concentrations in 20 and 40 ppm Ag-NP media, 0.041 and 0.037 ppm respectively. However, the specific growth rate was decreased in response to the nominal concentrations; 0.00881/hr at 20 ppm, and 0.00647/hr at 40 ppm Ag-NP.

It was concluded that the growth inhibition response of Lemna was actually related to the administrated Ag-NP concentration, even though the administrated Ag-NP might be additionally aggregated in test media to larger sizes.

Harris and Bali (2008)[29] investigated the limits of uptake and the distribution of Ag-NP in terrestrial plant *Brassica juncea* and *Medicago sativa*. In contrast to *B. juncea*, *M. sativa* showed an increase in Ag-NP uptake with a corresponding increase of concentration and exposure time. In this case, the exposure concentration of Ag-NP was 1,000-10,000 ppm. Because the growth of Lemna was completely inhibited at $\geq$100 ppm Ag-NP, an aquatic plant Lemna seems to be more sensitive species to nano-sized silver toxicity than the terrestrial plants studied previously.

Seeger *et al.* (2008)[30] tested the toxicity of two types of TiO_2-NP (25 nm in diameter; diameter $<$10 nm) on willow trees with the short-term test endpoints including transpiration, growth, and water use efficiency. No significant toxic effects to willow cuttings were found by TiO_2-NP at concentrations below 100 ppm.

Toxic effects of TiO_2-NP (100 nm) in two plant systems, *Allium cepa* and *Nicotiana tabacum* have been also observed. It was found that TiO_2-NP induced DNA damage, inhibition of the growth and increased lipid peroxidation in *A. cepa* root at 4 mM (calculated 319 ppm) and induced DNA damage in *N. tabacum* leaf at 2 mM (calculated 157 ppm)[31]. These results showed that the concentration range of TiO_2-NP toxicity to terrestrial plant (157 and 319 ppm) was comparable to the aquatic plant Lemna (LOEC 250 ppm) even though the endpoints of toxicity were different in those plants.

Currently very few data exist regarding observed environmental concentrations of Ag-NP and TiO_2-NP. Evidence that TiO_2-NP can leach from exterior facade paints and discharge into surface waters has been provided. The concentration of Ti element found in the surface runoff was as high as 600 μg/L[32]. Two studies modelled the quantities of TiO_2-NP released into the environment[33] and the predicted environmental concentrations were 0.002-16 μg/L in water compartment[24]. In this study, LOEC of TiO_2-NP for the growth inhibition of aquatic Lemna was calculated as 250 ppm, which was much higher than TiO_2-NP concentration of aquatic environment reported previously.

In this study, we analyzed aquatic toxicity caused by most abundantly used nanoparticles, Ag-NP and TiO_2-NP, using *L. paucicostata*. The EC50 of TiO_2-NP was nearly 40 times higher than Ag-NP, which

means the toxicity of Ag-NP for Lemna was much stronger than TiO_2-NP. The LOEC of TiO_2-NP might be over two orders higher than the environmental concentrations reported previously.

In case of Ag-NP, the LOEC value could not be compared to the environmental concentration, due to the lack of proper data. But the toxic response of Lemna was high enough to use and manage Ag-NP more carefully, and perform further investigation for the fate and concentration of Ag-NP in aquatic environment and organisms.

Materials and Methods

Plant Material and Culture Condition

Lemna paucicostata was selected for these studies. This plant was donated by Prof. T. J. Han, University of Incheon. Lemna was raised in 250 mL flask containing 100 mL AAP (algal assay proceduro) medium, pH 7.5, using reagent grade chemicals. The medium is then filtered through a 0.45 μm membrane filter into a sterile container. The photoperiodic regime was 16 hr light and 8 hr darkness, and a light intensity range was 6500-10000 lux[26]. Temperature was maintained at 25˚C.

Nanoparticles

Ag-NP was purchased from Nanopoly Co. (Seoul, South Korea) in colloidal form (5 mg/mL in water, 50 nm). TiO_2-NP was synthesized according to previously reported method[28]. Briefly, a mixture of 30 mL of titanium tetraisopropoxide (TTIP; Junsei 98%) and 5 mL of 2-propanol was added to 180 mL of distilled water and then 2 mL of nitric acid was added. The mixture was heated at 80˚C for 8 h, and then the solvent was evaporated using a rotavap to get the final product. The colloidal TiO_2-NP was formed by dispersion of the particles directly in distilled water or culture media for Lemna.

To characterize the nanoparticles, the colloidal Ag-NP and TiO_2-NP (dispersed in distilled water) were directly spotted onto 200-mesh copper formvar- and carbon-coated electron microscopy grids (Electron Microscopy Sciences, Hatfield, PA, USA) and viewed with a Hitachi HD-2300 scanning transmission electron microscope (TEM).

Toxicity Test and Endpoints

The test protocols were derived from the standard draft guideline 221 (OECD, 2002). Experiments were started by inoculating five fronds of Lemna in petri dish (100 mm × 15 mm) containing 50 mL of AAP culture media, supplemented with various concentrations of Ag-NP and TiO_2-NP. To prepare this test media, colloidal Ag-NP was as received from manufacturer and 5 mg/mL TiO_2-NP dispersed in culture media were diluted by AAP media to proper concentrations.

To determine the effects of the nanoparticels on growth of Lemna, it was raised for 7 days with three replicates. The plants were observed daily for toxicity symptoms (fronds number and chlorosis). After seven days, the number of fronds in each replicate was counted and recorded. This number was compared to the initial number of fronds and the specific growth rate (μ) was calculated according to the formula:

$$\mu = \frac{\ln n_t - \ln n_0}{t - t_0}$$

where n_t is the frond number at Day 0, n_0 is the frond number at Day 7, and $t - t_0$ is the test duration[26].

Data Analysis

Data were analyzed for normality and homogeneity of variances using Shaprio-Wilks and Bartlett's tests respectively, using Toxicalc Version 5.0 (Tidepool Scientific, 1996). If data met the assumptions, Dunnett's multiple comparison test was performed to determine which concentrations differed significantly from controls. Based on the statistical difference from the control value, the lowest observed effect concentration (LOEC) was determined.

The concentration of nanoparticles inhibiting the growth to 50% in the specific growth rate (EC50) was also calculated using Toxcalc Version 5.0. This method calculated EC50 values with corresponding 95% confidence intervals using linear or non-linear interpolation.

Determination of Nanoparticle Concentration in Culture Media

In order to estimate the change of nanoparticle concentrations in culture media during the test, 5 mL culture media was taken from the test vessels of 10, 20, 40 ppm Ag-NP and 31, 50, 100 ppm TiO_2-NP, on Day 0 and Day 7 of the experiment. The elemental concentration of Ag and Ti from water samples were checked by inductively coupled plasma atomic emission spectroscopy (ICP-AES, model: OPTIMA 4300DV). Each sample was filtered through 0.45 μm syringe filter (Whattman) prior to analysis to satisfy the requirement for instrumental management.

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