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ORIGINAL ARTICLE

Evaluation of platinum nanoparticles ecotoxicity using representatives of distinct trophic levels of aquatic biocenosis

Daniela Hlavkova [1], Miroslava Beklova [1], Pavel Kopel [2,3], Barbora Havelkova [1]

1 Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
2 Department of Chemistry and Biochemistry, Mendel University, Brno, Czech Republic
3 Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-61600 Brno, Czech Republic

Correspondence to: Daniela Hlavkova, MSc.
Department of Ecology and Diseases of Game, Fish and Bees
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackeho tr. 1946/1, 612 42 Brno, Czech Republic.
TEL: +420 5 4156 2660; E-MAIL: H16002@vfu.cz

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Abstract

OBJECTIVES: The aim of this study was to clarify the influence of three different sizes of platinum nanoparticles on aquatic ecosystem and assess the toxic effect in term of particle size. Tests were conducted on organisms representing all trophic levels of the aquatic ecosystem, namely producers (duckweed *Lemna minor*), consumers (water fleas *Daphnia magna*) and decomposers (bacteria *Vibrio fischeri*).
DESIGN: Experiments were carried out methodologically in accordance with the following standards: OECD 221 guideline (*Lemna* sp. Growth Inhibition test), OECD 202 guideline (Inhibition of the mobility of *Daphnia magna*) and ISO 11348-2 (Inhibitory effect of platinum nanoparticles on the light emission of *Vibrio fischeri*).
RESULTS: The most toxic have been the smallest sized platinum nanoparticles for all tested organisms. The highest toxicity of all tested samples (Pt1, Pt2, Pt3) was observed in bacteria ($30'EC_{50}$ = 135.47; 167.94; 254.64 $\mu g.L^{-1}$), respectively. The lowest toxicity was recorded for *Daphnia* ($48hEC_{50}$ = 405.74; 413.24; 514.07 $\mu g.L^{-1}$), respectively.
CONCLUSION: The ecotoxicity of platinum nanoparticles varies considerably according to the test organisms and particle size.

Abbreviations:

CI	- confidence interval
CV	- coefficient of variation
EC_{50}	- medium effective concentration is the concentration that causes an effect in test organisms amounting to 50% within a given exposure period when compared with the control
NMs	- nanomaterials
NPs	- nanoparticles
PtNPs	- platinum nanoparticles
PVP	- polyvinylpyrrolidones
SD	- standard deviation

INTRODUCTION

Nanoparticles are defined as particles whose structures have at least one of the dimensions in the range of 1 to 100 nm (Navarro *et al.* 2008a; Handy *et al.* 2008), or as particles which have a significantly different behavior compared to large particles based on size (Auffan *et al.* 2009). Nanotechnology and nanomaterials (NMs) play an important role in many key technologies for the last twenty years. NMs may provide solutions to technological and environmental problems in the field of solar energy, catalysis, pharmaceutical industry and water treatment (Mandal *et al.* 2006; Sharma *et al.* 2009). Extensive production and use of NPs may lead to their accidental release into aquatic, terrestrial and atmospheric environment, where they can have deleterious effects on a wide range of organisms (Navarro *et al.* 2008a). Therefore, some caution in the use of NMs should be taken until we find a reliable answer to the question if NMs do not pose an increased risk to humans and the environment (Navarro *et al.* 2008b; Griffitt *et al.* 2008).

Nanoparticles of platinum are of great scientific interest as they have many industrial and nanomedical applications (Bhattacharya & Murkherjees 2008; Pedone *et al.* 2017). PtNPs toxicity has been investigated for human cells, but only a few studies considering the effects of nanosized platinum on aquatic organisms (Asharani *et al.* 2011; Ksiazyk *et al.* 2015; SØrensen *et al.* 2016). The major sources of environmental contamination come from the immission by exhaust gases from motor cars equipped with catalytic converters and also from waste waters from hospitals, where the treatments of patients with cancer take place (Djingova *et al.* 2003; Ravindra *et al.* 2004; Supalkova *et al.* 2008; Rauch & Morrison 2008).

Toxic effects of NPs on the organisms depend on their chemical nature, shape and size. It's supposed, that higher toxicity in nanoparticles form compare to macroscopic form is caused by larger surface. As a rule, the smaller particles have a larger surface and are more toxic (Boyes *et al.* 2012; Dohnalova & Dohnal 2015).

The sustainability of the environment depends on effective forecasting and prevention of the influence of contaminants and consequently on the preservation of the biological chain (Bednarova *et al.* 2012). Aquatic plants are the most important primary producers of freshwater ecosystems that form the basis of aquatic food chains and balance the ecosystem by limiting or encouraging the spreading of animal populations (Jiang *et al.* 2012). Vascular plants of *Lemnaceae* family play an important role as bioindicators of ecological changes as well (Supalkova *et al.* 2008; Bednarova *et al.* 2012). *Daphnia magna* are widely used as test organisms to assess the acute toxicity of environmental contaminants (Martins *et al.* 2007). Informations about the toxic effect of nanoparticles on marine organisms compared to aquatic organisms are very limited, although they should not be neglected for several reasons. The behavior of nanoparticles in salty water will be considerably different from behavior in freshwater. Moreover, most industrial effluents end up in the seas, and nanoparticle manufacturers are often located on the coasts (e.g. Japan, China, United States). Coastal areas are the potential final disposal of all types of nanomaterials and therefore it should be given increased attention (Sovova & Koci 2012).

The aim of the present study was to assess the ecotoxicity of platinum nanoparticles by performing three bioassays on three different representatives of aquatic organisms and find out the toxic effect of PtNPs in terms of particle size.

MATERIAL AND METHODS

Test organisms

Daphnia magna and *Lemna minor* were obtained from an in-house culture collection of the Ecotoxicological laboratory of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The liquid dried bacteria *Vibrio fischeri* were commercially supplied (Hach-Lange GmbH in Dusseldorf, Germany).

Experimental design

Testing procedures were carried out in accordance with the following standards: OECD 202 (*Daphnia* sp. Acute Immobilisation Test), OECD 221 (*Lemna* sp. Growth Inhibition Test), ISO 11348-2 (The inhibitory effect of water samples on the light emission of *Vibrio fischeri*) with minor changes to fit our experimental conditions.

Inhibition of mobility of Daphnia magna

The immobilization test of *D. magna* was performed according to the OECD 202 guideline (CSN EN ISO 6341 – Determination of the inhibition of mobility of *Daphnia magna* Straus). Testing was performed with neonates (<24h), which were stored in 5 mL Eppendorf microtubes with dilution water with different concentrations of PtNPs. *Daphnia* specimens in the control were exposed to dilution water only. Test microtubes were maintained at temperature of 20±2 °C inside a controlled temperature chamber with a photoperiod (16 hours of light and 8 hours of dark) for 48 hours. At the end of the test the immobilization of daphnids was recorded. From the number of immobilized individuals at the test concentrations compared to control, the value of 48hEC_{50} (effect concentration) was determined using the probit analysis.

Lemna sp. – Growth Inhibition Test: microbiotest

The experiment was carried out as described in OECD 221 guideline (*Lemna sp*. Growth Inhibition test) in modification, using a microbiotest (Bednarova *et al.* 2014). Duckweed was allowed to grow in different concentration of PtNPs for 7 days under continuous warm fluorescent lighting (6,500–10,000 lx) at 24±2 °C. For

sample testing polystyrene macroplates with the lid were used. The volume of the test sample was 10 mL and at the beginning of the test five fronds of duckweed were added. We used three replicates for each test concentration and control. All visible fronds were counted every 24 hours and changes (e.g. frond size, chlorosis, necrosis) in plant development were observed.

The biomass size was determined by centrifuging the plants in plastic pre-weighed tubes at 3000 rpm for 10 minutes and then the tube was weighed again. The final biomass was determined by subtraction the tubes with biomass and pre-weighed tubes. Value of $168hEC_{50}$ was calculated using growth rate as an endpoint ($EC_{50\mu}$ = concentration that caused a 50% reduction in growth rate; EC_{50B} = concentration that caused a 50% reduction in biomass weight). For the test to be valid, we followed the criteria mentioned in the guideline.

Inhibitory effect on the light emission by Vibrio fischeri

The experiment was performed under the conditions specified in ISO 11348-2 (CSN EN ISO 11348-2 – Determination of the inhibitory effect of tested substances on the light emission of *V. fischeri*). Rehydrated bacteria were exposed to varying platinum nanoparticle concentrations dissolved in the dilution solution. Since *V. fischeri* is a marine organism, the test medium is a 2% NaCl solution. We used glass cuvettes with reactivated bacteria and prepared dilution series. Measurement was made using a luminometer unit equipped with a thermostat (LUMIStox 300, Hach-Lange, GmbH, Dusseldorf, Germany). The measurement period was 15 and 30 minutes. The values of 50% effective concentration (EC_{50}) were determined according to a valid standard. For the test to be valid, the value of the correction factor (f_{kt}) after 15 and 30 min of incubation has to be in the range from 0.6 to 1.8.

Chemicals

Listed chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), in ACS purity.

Platinum nanoparticles

The PtNPs with different sizes (Pt1: 3.1–10 nm); (Pt2: 4.2–21 nm) and (Pt3: 8.7–24.4 nm) were synthetized by reductive colloidal synthesis using polyvinylpyrrolidones (PVP) as a capping agent. $PtCl_4$ (0.07 g) with 37% HCL (33 µL) were dissolved in 10 mL of water. PVP (0.14 g) of different molecular weights (10 k, 21 k, 40 k) were dissolved in 40 mL of water. Solution (5 mL) of $H_2[PtCl_6]$ was added and then stirring for 1 hour. Then, 50 mg of $Na[BH_4]$ was added and filled into 50 mL with water and the solution was stirred for 2 hours (Buchtelova *et al.* 2017). For an objective assessment of the effect of PtNPs for aquatic organisms, nanoparticles were characterized by TEM (Tecnai F20, FEI, Eindhoven, Netherlands) and DLS (Zetasizer Nano ZS90, Malvern Instruments, Worcestershire, UK).

Analyzes were performed in phosphate buffered saline (pH 7.4). Prior to measurements, samples were incubated at 25 °C for 15 min.

All test solutions were prepared by diluting the initial dispersion of PtNPs in the culture medium to the desired concentration. Concentrations were selected on the basis of the range finding test. The tests were performed in three replicates at each concentration and controls. The concentration ranges of three PtNPs were the following for duckweed (50, 75, 100, 150 and 250 µg.L^{-1}) and for daphnia (25, 50, 100, 200, 300, 450 µg.L^{-1}). The concentration of Pt1–Pt3 for bacteria were (7.42, 14.84, 29.69, 59.38, 118.75, 237.5, 475, 950, 1900), (7.03, 14.06, 28.13, 56.25, 112.5, 225, 450, 900, 1800 µg.L^{-1}) and (9.38, 18.75, 37.5, 75, 150, 300, 600, 1200, 2400 µg.L^{-1}), respectively.

Statistical analysis

The $168hEC_{50}$ values were calculated using the TOXICITA 3.1 software (VUV Ostrava, Czech Republic) by means of regression analysis of the data with 95% confidence interval (CI), based on squared deviations of experimental values from the selected approximation function.

The $48hEC_{50}$ values, as well as their associated 95% confidence intervals, were determined by probit analysis using a computer program (PROBITY VURH, Vodnany, Czech Republic).

RESULTS AND DISCUSSION

To determine the size-effect of nanoparticles, we produced PtNPs of different particle sizes. PtNPs were characterized for their morphology and size distribution (Figure 1). Synthesized PtNPs had irregular, polyhedral shape. Based on the performed particle size analysis it was found that more than 75% of PtNPs1 were within the size range 4.2–5.6 nm, while nearly 80% of PtNPs3 were within the range 11.7–18.2 nm.

In this study, we investigated differential toxicity of various-sized PtNPs on three species representing different levels of an aquatic trophic chain. The influence of selected PtNPs on the observed organisms is shown in Figures 2–5. The results EC_{50} of PtNPs on the tested organisms are shown in Table 1.

The results from the present study demonstrate that nanoparticles are capable of causing acute toxicity in multiple aquatic species. However, toxicity differs significantly with the particle sizes and the tested organisms. In the species tested, PtNPs were toxic with $48hEC_{50}$ of less than 520 µg.L^{-1} in daphnia, $30´EC_{50}$ of less than 260 µg.L^{-1} for bacteria and $168hEC_{50}$ (biomass) less than 250 µg.L^{-1} for the macrophyte (Table 1).

The results of this *Daphnia magna* experiment were difficult to assess because of the lack of published data to compare with. There are recently published papers about the ecotoxicity of platinum nanoparticles, where authors studied the impact e.g. on *Sinapis alba* and

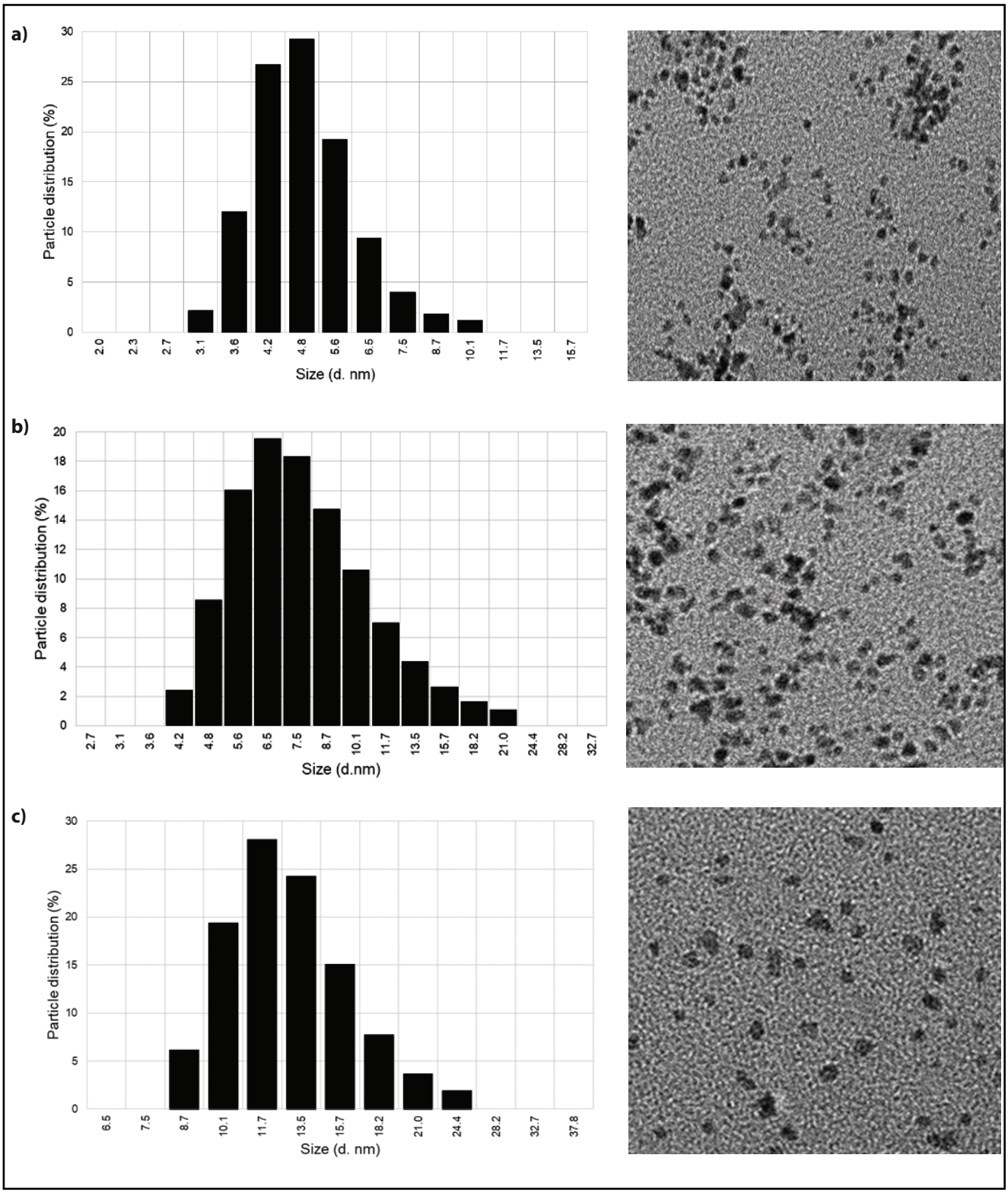

Fig. 1. TEM micrographs and particle size histograms of investigated PtNPs: (a) PtNPs-1 (3.1–10 nm); (b) PtNPs-2 (4.2–21 nm); PtNPs-3 (8.7–24.4 nm).

Lepidium sativum plants (Asztemborska *et al.* 2015), *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* (SØrensen *et al.* 2016) and zebrafish embryos (Asharani *et al.* 2011). The 48hEC_{50} value of PtNPs1 for *Daphnia magna* was 405.74 μg.L^{-1}. Since *Daphnia* are part of the diet of other organisms (e.g. fish), there is a potential for uptake and subsequent transfer to higher organisms.

Our microbiotest compared the effect of PtNPs on the vegetative growth of duckweed colonies. During

Tab. 1. Summary values of EC_{50} of PtNPs (confidence interval is calculated for normal distribution and a level of 95%).

Test organisms	Platinum nanoparticles - size (nm)		
	Pt1 (3.1–10)	**Pt2 (4.2–21)**	**Pt3 (8.7–24.4)**
***Daphnia magna* 48hEC_{50} (µg.L^{-1})** **95% CI**	405.74 (291.28–568.97)	413.24 (360.17–474.19)	514.07 (292.08–928.43)
***Lemna minor* 168hEC_5 (µg.L^{-1})** **95% CI**	10.67 (a)	50 (a)	121.90 (a)
***L. minor* (biomass) 168hEC_{50} (µg.L^{-1})** **95% CI**	140.77 (140.77–140.77)	247.81 (247.81–247.81)	249.37 (249.37–249.37)
***Vibrio fischeri* 15´EC_{50} (µg.L^{-1})**	166.06 fkt 0.97	166.68 fkt 0.96	274.80 fkt 0.87
***Vibrio fischeri* 30´ EC_{50} (µg.L^{-1})**	135.47 fkt 1.01	167.94 fkt 0.98	254.64 fkt 0.89

95% CI – 95% confidence interval; fkt – correction factor for 30 minutes incubation must be between 0.6–1.8; [a] Not obtainable

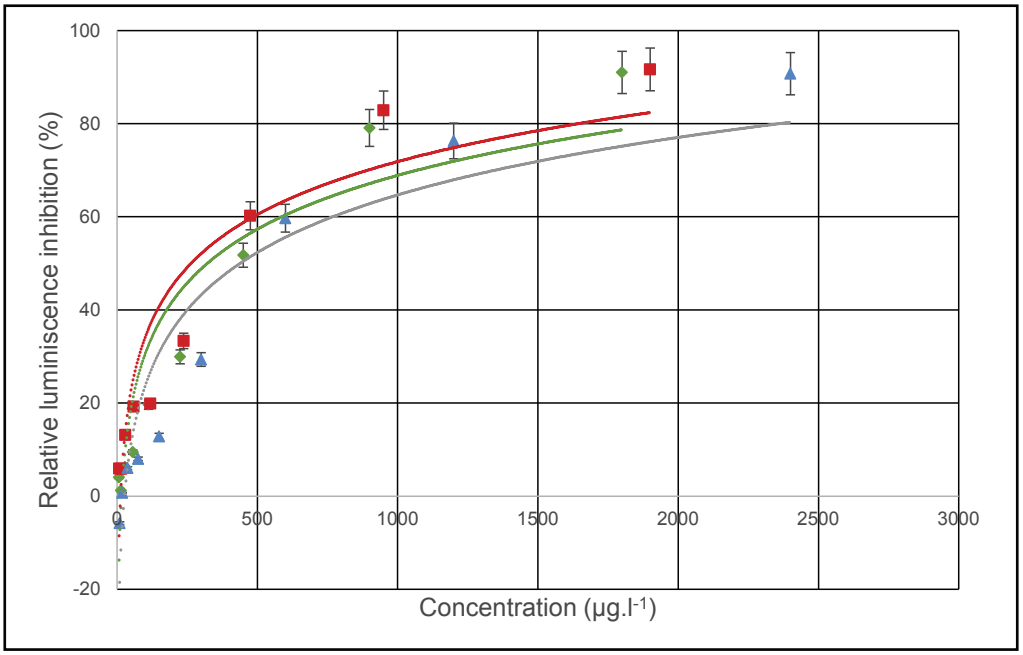

Fig. 2. Dose-response curves of Pt1 (■), Pt2 (♦) and Pt3 (▲) after 15 minutes of incubation for *Vibrio fischeri*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

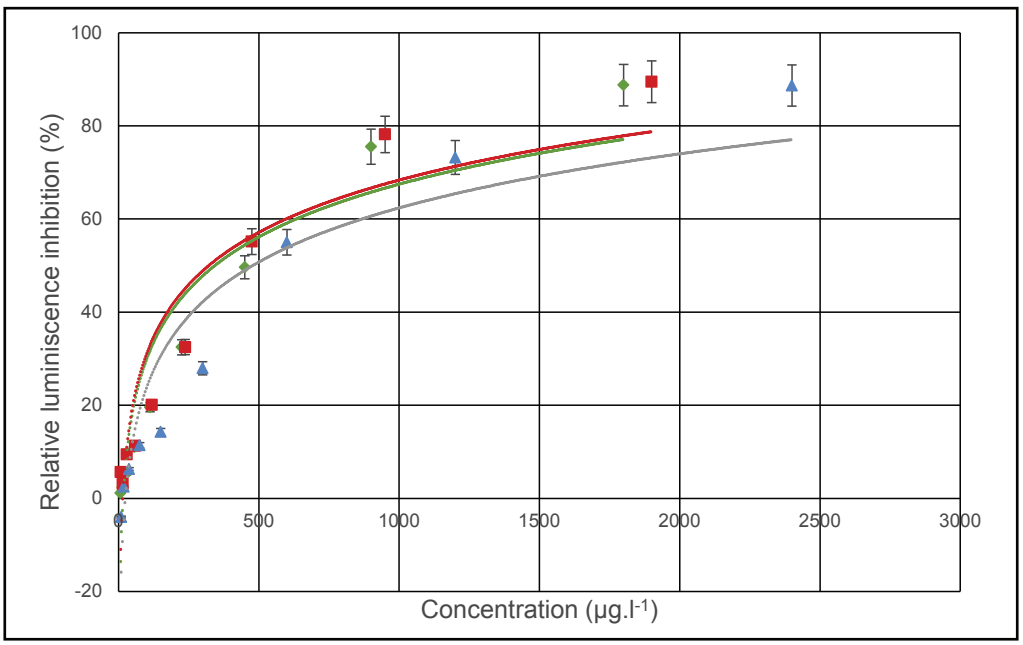

Fig. 3. Dose-response curves of Pt1 (■), Pt2 (♦) and Pt3 (▲) for *Vibrio fischeri* after 30 minutes of incubation. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

the experiment, we observed changes in the appearance of the leaves. Plants began to turn yellow (chlorosis), and at the end of the experiment we observed leaves with white areas (necrosis). In addition, the individual colony size and root length decreased with the increasing PtNPs concentration. Rooted growth, chlorosis and necrosis are some visible signs indicating severe metal phytotoxicity (Rahman *et al.* 2011; Bednarova *et al.* 2014).

For PtNPs after 7 days exposure there was no significant effect of dose upon the frond number. The experiment with duckweed showed 5% of growth inhibition and the values of $168hEC_{5\mu}$ for Pt1–Pt3 were 10.67 $\mu g.L^{-1}$, 50 $\mu g.L^{-1}$ and 121.90 $\mu g.L^{-1}$, respectively. Ksiazyk *et al.* (2015) investigated the toxicity of uncoated PtNPs (30–60 nm) to *Pseudokirchneriella subcapitata* (72-h test) using the algal growth inhibition test and obtained the EC_{50} many times higher than our result to *L. minor*. This difference could be related to the different tested organism and particle size. In our study the particles were up to six-fold smaller. It is also known that the toxicity of NPs varies with different capping agents (Chung *et al.* 2008). Another study examining effects of TiO_2NPs on *Lemna minor* has revealed no effects on plant growth (Picado *et al.* 2015). After 7 days the biomass weight was significantly lower in plants exposed to 100, 150 and 250 $\mu g.L^{-1}$ than in the control for all three PtNPs (Figure 5). The morphology

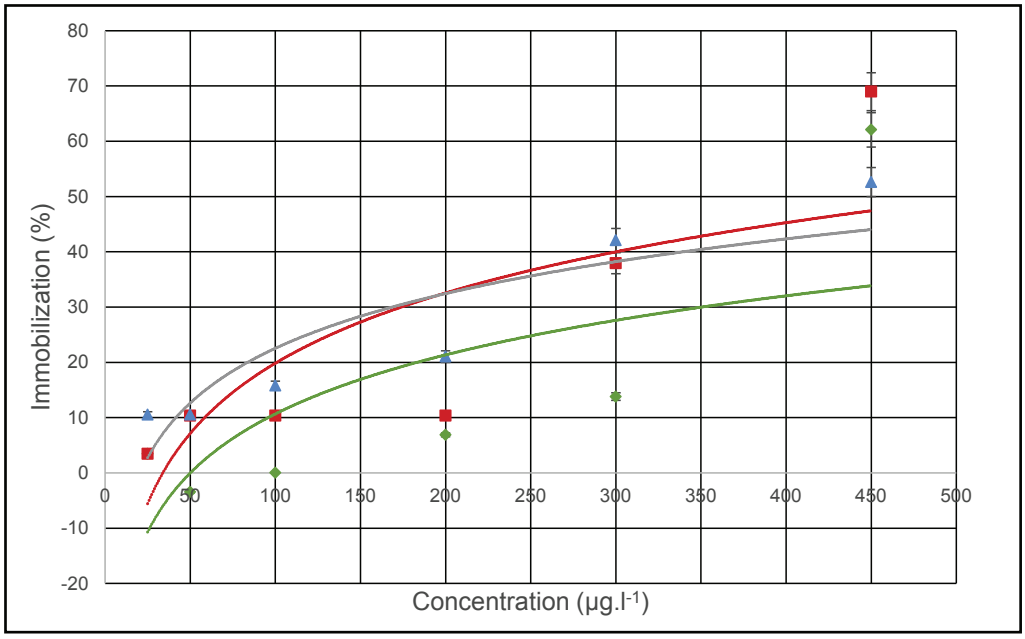

Fig. 4. Dose-response curves of Pt1 (■), Pt2 (♦) and Pt3 (▲) for *Daphnia magna*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

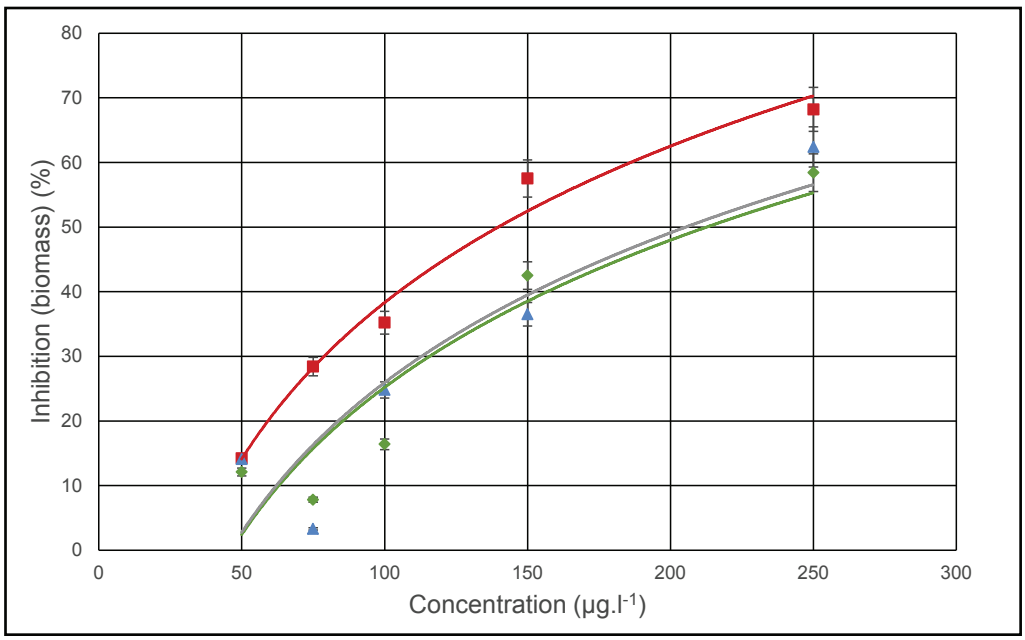

Fig. 5. Dose-response curves of Pt1 (■), Pt2 (♦) and Pt3 (▲) for *Lemna minor*. Error bars correspond to 95% confidence intervals. Dotted lines represent the fitting to the median effect equation.

of leaves was changed generally from the third day of the incubation, while the inhibition of the vegetative growth was evident after 6 days. In the present study, the final biomass was a more sensitive parameter than the frond number. Radic *et al.* (2009) observed that the frond number and the final biomass were almost equally sensitive parameters.

The toxic effects of exposure to PtNPs for marine organisms were tested using bacteria *Vibrio fischeri*, after contact time of 15 and 30 min. As shown in Table 1, the concentration of PtNPs in contact with *V. fischeri,* killed more bacteria after 30 min than after 15 min and led to higher light inhibition and higher toxicity. Tests with bacteria confirmed the results of a study by Binaeian *et al.* (2012) that they need more time to diffuse to the cells and degrade lipids, carbohydrates, proteins and DNA.

The bacteria were the most sensitive to nanoparticles from all three organisms. It can be stated that bacteria can be used as biosensors for rapid and low-cost detection of acute toxicity of nanomaterials (Binaeian *et al.* 2012; Binaeian & Soroushnia 2013).

CONCLUSION

In this study, we have reported the toxicity of platinum nanoparticles on crustacean *Daphnia magna*, the macrophyte *Lemna minor* and bacteria *Vibrio fischeri*. The tested PtNPs were found to be harmful to aquatic life at low concentrations ($\mu g.L^{-1}$). According to the EC values, the toxicity decreased in the following order: Pt1>Pt2>Pt3. The size hypothesis can be confirmed for PtNPs. Considering the toxicity along with the extensive exposure potential of PtNPs, additional hazard and risk assessment is crucial.

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