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Toxicity of various silver nanoparticles compared to silver ions in the Ponto-Caspian amphipod *Pontogammarus maeoticus* (Sowinsky, 1894)

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ABSTRACT
According to the increased probability of the presence of nanomaterials in the aquatic ecosystems, the present study examined the toxicity of three engineered silver nanoparticles (AgNPs) as well as silver ions in the *Pontogammarus maeoticus*, a brackish water benthic organism living in the littoral zone of the Caspian Sea. The animals were acutely exposed to different concentrations of two commercially prepared colloidal forms and a freshly prepared suspension of silver nanoparticles, plus $AgNO_3$ during 48 hr. The number of mortalities was assessed and lethal concentration values were calculated using the EPA Probit Analysis Program. According to median lethal concentrations (LC_{50}), the order of sensitivity of this amphipod to tested silver compounds was as: previously prepared AgNPs colloids > freshly prepared AgNPs suspension > $AgNO_3$. Also the signs of nanoparticle accumulation were evident between the pereopods and pleopods of this gammarid; this accumulation could be one of the reasons for the higher toxicity of silver nanoparticles in comparison with silver ions in *P. maeoticus*. More acute and chronic studies are needed to understand the various aspects of nano-silver toxicity on amphipods in different salinities.

Key words: Aquatic Nanotoxicology, Caspian Sea, Gammaridae, Silver Nanoparticles, Colloid.

INTRODUCTION
Specific physical and chemical properties of nanomaterials caused more than ever tend to use them in human life. The statistics indicates that nanotechnology consumer products inventory contains 1628 products or product lines in 26 countries till October 2013 (Woodrow Wilson Database, 2014). Among them, silver nanomaterials with 383 products (about 23.5%) are the most common nanomaterial mentioned in the product descriptions. According to estimations, by 2020 there will be about $3 trillion in products that incorporate nanotechnology as a key performance component (Roco, 2011).

Despite all the advantages of nanomaterials in improving life and livelihoods of people, they may also cause risks to humans and the environment. That is why it is important to recognize the adverse effects of nanomaterials, an issue which is addressed in "nanotoxicology". In addition, since part of the engineered nanomaterials produced globally will be ended up in water bodies (0.4–7 % according to Keller *et al.* 2013), understanding the effects of these substances on aquatic organisms is very important, an issue which is addressed in "aquatic nanotoxicology". The presence of nano silver in aqueous environments has been predicted to range from 0.03 to 0.32 micrograms per liter (Mueller & Mowack 2008; O'Brien & Cummins 2010). Moreover, an estimated 63 tons of nano silver enter to aquatic systems annually on a worldwide basis (Keller *et al.* 2013). Therefore, understanding the effects of nano silver on aquatic organisms is critical.

There are several recent publications about toxic effects of some nanomaterials (including Au, ZnO, CuO, NiO, TiO_2, CeO_2, and quantum dots nano-particles, as well as single- and multi-walled carbon nano-tubes and silicon carbide nano-wires) on amphipods (Kennedy *et al.* 2008; Bundschuh *et al.* 2011; Mwangi *et al.* 2011; Fabrega *et al.* 2012; Jackson *et al.,* 2012; Hanna *et al.,* 2013; Poynton *et al.* 2013; Kalcíková *et al.* 2014; Li *et al.* 2014a,b; Park *et al.* 2014, 2015; Garaud *et al.* 2015; Revel *et al.* 2015; Rosenfeldt *et al.* 2015); Most of these studies show that these sediment - dwelling organisms are likely to have a high potential exposure and are highly susceptible to the effects of nanomaterials and should be considered in the risk assessment of these substances.

The family Gammaridae belonging to order Amphipoda are found throughout a diverse range of freshwater, coastal and brackish environments and are generally considered as macrophagous herbivores/detritivores. The *Pontogammarus maeoticus* (Sowinsky 1894) has a Ponto-Caspian distribution area which covers the Caspian, Azov, and Black Seas (Barnard & Barnard 1983; Stock *et al.* 1998).

In the brackish water of Iranian coasts of the Caspian Sea, this benthic infauna species is widely abundant and distributed (Mirzajani, 2003) and usually feed on detritus.

This aquatic organism itself is an important prey for many commercial fish of the Caspian Sea, including sturgeons. To our knowledge no information is available in the case of silver nanoparticles toxicity in aquatic amphipods. We have previously studied the acute toxicity of three types of well characterized silver nanoparticles in *Daphnia magna*, as a model freshwater organism, and showed that each of them represent a specific amount of toxicity which is related to the chemical characteristics and aggregation of the different Ag nanoparticles (Asghari *et al.* 2012). In the present study we examined the toxicity of those silver nanoparticles including two commercially prepared colloidal forms and a freshly prepared suspension as well as silver ions in the *Pontogammarus maeoticus*, as a brackish water organism.

MATERIALS AND METHODS

2.1. Nanoparticles and characterization

Three kinds of well characterized nano silver (AgNPs) including two types of colloidal silver nanoparticles and a suspension of silver nanoparticles were used in this study.

The colloidal forms (AgNPs-1 & AgNPs-2) were commercially prepared by ABC Nanotech Co. LTD (Daejeon, South Korea) and Nano Nasb Pars Co. Ltd (Tehran, Iran), respectively. The suspension form (AgNPs-3) was freshly prepared from a silver nano-powder bought from Xuzhou Hongwu Nanometer Material Co. Ltd (Jiangsu, China) and suspended in distilled water by sonication method exactly as described in Asghari *et al.* (2012).

Detailed characterizations of each of these silver nanoparticles could be found in Asghari *et al.* (2012) and are also briefly shown in Table 1.

In addition, to compare the toxicity of the different silver nanoparticles with that of silver ions, $AgNO_3$ (purity > 99.5%, Fluka Chemika, Sigma-Aldrich, Switzerland) was used as the source of silver ions.

2.2. Test organisms

P. maeoticus were collected one week before the start of the experiment from the southern coast of the Caspian Sea close to Noor City (36° 35' 1.8'' N, 52° 2' 32.8'' E, Mazandaran, Iran), far from any settlement and agricultural activity to avoid possible contamination.

In the laboratory, the gammarids were kept in aerated sea water at 12 $g.L^{-1}$ salinity (salinity of their living area in the south of the Caspian Sea) at a constant temperature of 20 ± 1 ºC and fed ad libitum with lettuce leaves until the start of the experiment.

To minimize the effect of body size on the results of the experiments, only adults with a body length between 8 and 10 mm were used for toxicity tests.

2.3. Experimental design

The exposures of test organisms were done in 200 ml glass beakers containing 10 organisms and 150 ml of freshly prepared test solution.

All the tests were conducted in a water bath system with a constant temperature (20 ± 1 °C) and 16 h light: 8 h dark photoperiods. Feeding of organisms was stopped 6 hours before beginning of the toxicity tests and the animals were not fed during the bioassays. In this study, fully aerated sea water were used as the exposure media and the test solutions were prepared immediately prior to use by diluting the different stocks of silver nanoparticles or silver ions in the sea water. After adding appropriate amounts of the stocks to the sea water, the stock mixtures were stirred using a magnetic stirrer to distribute the suspension at a stable concentration. Series of preliminary experiments conducted to determine the range of chemical concentrations that caused mortality in *P. maeoticus*. According to the determined concentration ranges, effective concentrations were then selected for each substance (Table 2). Each bioassay included completely random design, consisting silver nanoparticle treatments and their controls in triplicate. To evaluate the toxicity of each chemical, the mortality of the gammarids in each test beaker assessed at 24 h and 48 h.

Table 1. Characterizations of the nanomaterials used (derived from Asghari *et al.* 2012).

	Chemical notation	**AgNPs-1**	**AgNPs-2**	**AgNPs-3**	
	Brand	SARPU 200 KW	Nanocid L2000	-	
	Manufacturer	ABC Nanotech Co., LTD (Daejeon, Korea)	Nano Nasb Pars Co., Ltd (Tehran, Iran)	Xuzhou Hongwu Nanometer Material Co., Ltd (Jiangsu, China)	
	Appearance	Blackish-brown Colloid	Yellowish-brown Colloid	Black powder (Then suspended)	
Manufacturer Information	Size	5-25	16.6	20	
	Purity	99.98%	-	99%	
	Capping agent	1.0 wt% citrate	-	-	
				Dry powder	Suspension
	Concentration (mg.L^{-1})	200000	4000	-	400
Information from TEM	Max. Diameter (nm)	15.83	129	161	About 70.31% of the aggregates had diameters from 25 to 100 nm, while most of the others had diameters from 100 to about 250 nm.
	CMD* (nm)	7.32	6.47	17.97	
	GMD* (nm)	7.96	12.65	14.39	
	GSD*	1.35	1.46	1.31	
	Shape	Spherical	Spherical	Spherical	Large aggregates
	pH	5.8	2.4	-	7.32

*CMD: Count median diameter, GMD: Geometric mean diameter, GSD: Geometric standard deviation.

Table 2. Concentration gradients of different nanoparticles and $AgNO_3$ used for acute toxicity tests (concentration ranges were selected according to the preliminary experiments).

Chemical notation	**Concentration (mg.L^{-1})**					
AgNPs-1 colloid	10	25	50	100	150	200
AgNPs-2 colloid	5	10	25	50	75	100
AgNPs-3 suspension	10	25	50	100	150	200
$AgNO_3$ solution	10	25	50	100	150	200

2.4. Statistical analysis

The 48-h lethal concentration values (LC_{10}, LC_{50}, and LC_{90}), as well as their associated 95% confidence intervals (95% CI) were calculated using the US EPA Probit Analysis Program (version 1.5). In required cases, statistical analyses were carried out using standard ANOVA techniques, followed by Tukey's significant difference test (SPSS Ver. 17.0).

RESULTS

During the experiments, the mean and SD of the water pH and dissolved oxygen in the exposure vessels were 8.3 ± 0.1 and 8.4 ± 0.3 mg.L^{-1}, respectively. Also, there was no significant difference between treatments in this regard ($P > 0.05$). In all concentrations of all three groups of silver nanoparticle (AgNPs-1, AgNPs-2 and AgNPs-3), formation of brownish sediments was visible on the bottom of the test vessels. The addition of silver nitrate to the sea water did not cause visible sedimentation. During the exposure period, the mortality in the control groups was less than 10% for all the tests. The lowest concentrations of AgNPs-1, AgNPs-2, AgNPs-3 and $AgNO_3$ which caused 100% mortality in gammarids after 48 hours were 200, 100, 200, and 200 mg.L^{-1}, respectively. Also the highest concentrations of AgNPs-1, AgNPs-2, AgNPs-3 and $AgNO_3$ which did not cause any mortality in gammarids during 48 hours were 5, 1, 5 and 5 mg.L^{-1}, respectively.

The average values of the lethal concentrations and their 95% confidence limits are shown in Table 3. The median lethal concentrations of AgNPs-1, AgNPs-2, AgNPs-3 and $AgNO_3$ were calculated as 25.026, 27.135, 38.240 and 100.651 mg.L^{-1}, respectively.

Although the LC_{50} of AgNPs-1 and AgNPs-2 was statistically similar ($P > 0.05$), but its value for $AgNO_3$ was statistically higher than AgNPs-3, and its amount for AgNPs-3 was higher than AgNPs-1 and AgNPs-2 ($P < 0.05$) as shown in Fig. 1. In the case of gammarids exposed to silver nanoparticles, large amounts of dark materials were observed between the walking legs (pereopods) and swimming legs (pleopods) which are probably the signs of nanoparticle accumulation.

Table 3. Lethal-concentration values, with lower and upper 95% confidence limits (CL), of different nanoparticles and $AgNO_3$ for *Pontogammarus maeoticus* during 48 h.

Chemical notation	Average LC_{10} (95%CL) (mg.L^{-1})	Average LC_{50} (95%CL) (mg.L^{-1})	Average LC_{90} (95%CL) (mg.L^{-1})
AgNPs-1 colloid	4.048 (3.749-5.030)	25.026 * (23.569-28.975)	154.710 (140.467-158.572)
AgNPs-2 colloid	1.575 (1.028-2.155)	27.135 * (24.974-28.378)	489.592 (387.303-557.125)
AgNPs-3 suspension	7.241 (5.596-10.406)	38.240 ** (32.339-42.405)	201.949 (176.752-235.718)
$AgNO_3$ solution	48.853 (20.173-69.279)	100.651 *** (72.063-121.729)	207.369 (166.893-219.916)


Fig. 1. Photograph of *Pontogammarus maeoticus* from the control group (left) and 100 mg.L^{-1} colloidal silver nanoparticles (AgNPs-1, right). Signs of nanoparticle accumulation can be seen between the walking and swimming legs.

DISCUSSION

Results of our previous study on *D. magna*, showed that silver nano powder subsequently suspended in exposing water (AgNPs-3) was much less toxic than previously prepared nano Ag colloids (AgNPs-1 and AgNPs-2), while colloidal AgNPs and silver nitrate were almost identical in terms of their toxicity (Asghari *et al*. 2012) The comparative toxicity results for the different silver nanoparticles and $AgNO_3$ used in the current study suggest that AgNPs-3 suspension was relatively less toxic than AgNPs-1 and AgNPs-2 colloids to *P. maeoticus*; also all three types of silver nanoparticles were more toxic than silver nitrate. Also when generally comparing the results, we find that these silver compound are much more toxic to *D. magna* than *P. maeoticus*. Difference in obtained toxicity results may be due to the physiological differences between these two species, which affects their response mechanisms to these chemicals. Also the *P. maeoticus* lives in higher salinities than *D. magna* and it has been shown previously that salinity can greatly affect aquatic toxicity of silver nanoparticles (Salari Joo *et al.* 2013). Many studies have shown that the toxicity of silver ions is higher than silver nanoparticles (i.e. Caballero-Díaz *et al.* 2013; Christina *et al.* 2012; Waalewijn-Kool *et al.* 2014) and even some researchers believe that ions, not particles, are responsible for the toxicity of silver nanoparticles (Xiu *et al.* 2012). Although we still do not know exactly why the toxicity of silver nanoparticles to *P. maeoticus* was higher than silver nitrate, but based on the observations, it may be due to the higher accumulation capacity of silver nanoparticles in this organism which can be due to its filter-feeding ability; this feature makes the nanoparticles to be trapped in the body parts of the animal, while this condition does not apply in the case of ions. Future studies are needed to compare the quantity of bioaccumulation of silver nanoparticles and silver ions in *P. maeoticus*; It helps to more accurate judgments about the effect of accumulated silver on the toxicity of each of these silver compounds.

CONCLUSION

To our knowledge, the present study was the first research that was conducted on the effects of silver nanoparticles on an aquatic amphipod. Overall, the results of this study revealed that both silver nanoparticles and $AgNO_3$ have some degrees of acute toxicity in the Ponto-Caspian amphipod, *P. maeoticus*. Although concentrations causing acute toxicity in this brackish water organism are higher than those needed for most studied freshwater organisms, but this does not mean that these materials are safe to be released to the Caspian Sea or any other brackish water aquatic habitats. More acute and chronic studies are needed to investigate the effects of silver nanoparticles on aquatic amphipods in different salinities including fresh, brackish or saline ecosystems.

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مقایسه سمیت انواع مختلف نانوذرات نقره با یون نقره در گاماروس دریاچه خزر (*Pontogammarus maeoticus*)

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چکیده

در پی افزایش احتمال حضور نانومواد در بوم‌سازگان‌های آبی، در مطالعه حاضر سمّیت سه نوع مختلف نانوذرات نقره و همچنین یون نقره، در گاماروس دریاچه خزر *Pontogammarus maeoticus* به عنوان یک جانور کفزی ساکن آب‌های لب‌شور مناطق ساحلی دریاچه خزر، مورد بررسی قرار گرفت. بدین منظور، گاماروس‌ها طی آزمون‌های حاد ۴۸ ساعته در معرض غلظت‌های مختلفی از دو نوع کلوئید تجاری نانوذرات نقره، یک نوع سوسپانسیون تازه تهیه شده نانوذرات نقره، و همچنین نیترات نقره قرار گرفتند. پس از بررسی تعداد مرگ و میر، مقادیر غلظت‌های کشنده با استفاده از نرم افزار پروبیت محاسبه گردید. بر اساس مقادیر محاسبه شده‌ی غلظت‌های کشنده میانی (LC50)، بیشترین حساسیت این گونه‌ی ناجورپا به ترکیبات نقره مورد بررسی، به ترتیب عبارت بود از: کلوئیدهای از پیش تهیه شده نانوذرات نقره > کلوئید تازه تهیه شده نانوذرات نقره > نیترات نقره. همچنین نشانه‌هایی از انباشتگی نانوذرات در بین پاهای شنا و پاهای حرکتی گاماروس‌ها قابل مشاهده بود که این انباشتگی می‌تواند یکی از دلایل بالاتر بودن سمّیت نانوذرات نقره در مقایسه با یون نقره در گونه مورد مطالعه باشد. انجام آزمون‌های حاد و مزمن بیشتر، به منظور مطالعه جنبه‌های مختلف سمّیت نانوذرات نقره در ناجورپایان در شوری‌های مختلف ضروری به نظر می‌رسد.

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