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Toxicity of pure silver nanoparticles produced by spark ablation on the aquatic plant *Lemna minor*

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A B S T R A C T

The increasing penetration of nano-products to the market is raising big concerns about the potential toxic and environmental effects of their constituent engineered nanoparticles (ENPs). Contradictory toxicity test results reported in the literature thus far can be explained by differences in the ENP production methods, which can strongly affect nanoparticle purity and therefore the outcome of the tests. In this paper we investigate the toxicity of Ag nanoparticles (AgNPs) produced by spark ablation - a gas-phase technique that can deliver well-defined nanoparticles of high purity - on *Lemna minor*. Our results show that AgNPs exhibit a toxic behavior at concentrations as low as 5 μg L^{-1}, which is considerably lower compared to the threshold concentrations reported in other studies. This difference can be attributed to the purity of the ENPs used in our measurements, which can release higher concentrations of toxic Ag^{+} ions upon dilution in the test solutions.

1. Introduction

Engineered nanoparticles (ENPs) are designed to have dimensions in the nanometer scale (i.e., smaller than 100 nm) in order to exhibit physical and chemical properties that differ from those of their large-particle counterparts and bulk materials (Handy et al., 2008; Wang, Wick, & Xing, 2009). The properties of ENPs that give nanomaterials and nanoparticle-containing products their unique characteristics may very well be responsible for their toxic effects when released to the environment (cf. Biskos & Schmidt-Ott, 2012; Dwivedi et al., 2015; Kumar et al., 2014; Mueller & Nowack, 2008; Nowack & Bucheli, 2007; Maynard et al., 2006).

ENPs are currently being used in an increasing number of products including cosmetics, personal care products and dietary supplements, which are already available in the global market (Klaine et al., 2008; Kreuter, 2007). Despite their widespread use, however, evaluations of the associated environmental and health risks is lagging far behind (Kalantzi & Biskos, 2014). One way to assess these risks is to study their toxic effect on sensitive plants. Tests using the aquatic plant *Lemna minor* are widely used to determine the impacts for a wide range of substances released to the environment (Moody & Miller, 2005), and recent modifications of the methods have been used to assess the toxic effects of ENPs.

Oukarroum, Barhoumi, Pirastru, and Dewez (2013) showed that commercially available Ag nanoparticles (AgNPs), at concentrations above 100 μg L^{-1}, exhibit toxic effects on *Lemna gibba* due to the production of intracellular reactive oxygen species. Along the same lines, Gubbins, Batty, and Lead (2011) showed that AgNPs produced by classical wet-chemistry methods reduce the number of grown fronds on *Lemna minor* at concentrations above 80 μg L^{-1}, while ionic silver exhibits similar effects at concentrations as low as 5 μg L^{-1}. More recently, Üçüncü et al. (2014) reported that AgNPs produced by laser ablation in the liquid phase, can significantly affect the growth of *Lemna minor* at concentrations as low as 8 μg L^{-1}. The difference between the threshold

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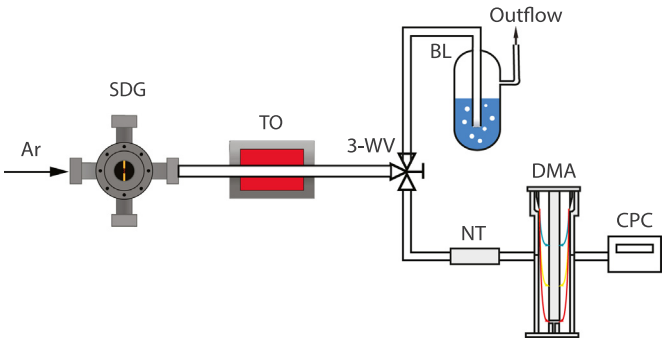

Fig. 1. Schematic layout of the experimental setup used for the synthesis and characterization of the test AgNPs. Key: SDG, spark discharge generator; TO, tube oven; 3WV, three-way valve; BL, bubbler; NT, aerosol neutralizer; DMA, differential mobility analyzer; CPC, condensation particle counter.

concentrations reported by Üçüncü et al. (2014) and those reported by Gubbins et al. (2011) and Oukarroum et al. (2013), indicates that the outcome of these tests is strongly dependent on the production method of ENPs, which can significantly affect their level of purity.

ENPs employed in toxicity tests are commonly synthesized by traditional wet-chemistry methods, which often introduce impurities on their surface. In contrast, aerosol-based techniques can provide ENPs of the highest possible purity (Biskos, Vons, Yurteri, & Schmidt-Ott, 2008). These techniques can provide good control over the size of the nanoparticles, providing another feature that is very attractive for a number of applications including toxicity tests.

In this work we investigate the toxicity of high-purity silver nanoparticles, having diameters from 10 to 80 nm, generated by spark ablation in the gas phase, on the aquatic plant *Lemna minor* .

2. Materials and methods

2.1. Production of AgNPs

The AgNPs used in our tests were synthesized by spark ablation; a gas-phase method that produces nanoparticles of very high purity and provides good control over their size, morphology and composition (Fig. 1; Feng, Biskos, & Schmidt-Ott, 2015; Pfeiffer, Feng, & Schmidt-Ott, 2014; Tabrizi, Ullmann, Vons, Lafont, & Schmidt-Ott, 2009). In this technique, repeated spark discharges formed between two Ag electrodes produce metal vapor clouds at ambient pressure. The vapor clouds are subsequently cooled down to room temperature forming AgNPs having diameters from those of a few atoms (cf. Maisser, Barmpounis, Attoui, Biskos, & Schmidt-Ott, 2015), and up to a few tens of nanometers (Feng et al., 2016). The cooling of the vapors is aided by a high purity N_2 flow that passes between the electrodes, carrying also the nanoparticles out of the reactor. Because the particles produced by spark ablation are highly agglomerated, we passed them through a tube oven maintained at 900 °C to "melt" them to spherical particles (cf. micrographs provided by Kourmouli et al., 2018).

The size distribution of the resulting particles was measured using an electrical mobility spectrometer consisting of an aerosol particle charge neutralizer (NT; Wiedensohler, 1988), a differential mobility analyzer (DMA; Knutson & Whitby, 1975), and a condensation particle counter (CPC; Agarwal & Sem, 1980), as shown in route 1 of Fig. 1. The resulting particles had a spherical shape and diameters in the range of 10–80 nm, with a geometric mean at ca. 30 nm (cf. Fig. 2), falling in the same size range with those used in earlier studies (Gubbins et al., 2011; Üçüncü et al., 2014). After the size distribution of the particles was determined, the aerosol stream was passed through a bubbler and the particles were captured in de-ionised water as shown in route 2 of Fig. 1. The resulting aqueous nanoparticle-containing solutions were then used in the toxicity tests described below.

2.2. Lemna minor sensitivity assays

A stock culture of *Lemna minor* in agar was purchased from the Federal Environmental Agency (UBA, Germany) and grown according to the ISO 20079 (2005) guidelines. The growth of the plants was made in a glass Pyrex vessel with modified Steinberg medium at a pH of 5.5 ± 0.2 (ISO 20079, 2005) in non-axenic conditions. For all the toxicity tests we used sterile APHA medium to avoid contamination with algae and microorganisms. The plants were acclimatized in the APHA medium for 16 h before starting the test.

After plant acclimatization, 12 fronds with 3–4 leaves each were placed in separate vessels containing 100 ml of the test medium with silver nanoparticles at concentrations of 0 (control), 5, 10, 20, and 40 μg L^{-1}. According to the ISO 20079 protocol, the pH of the test medium was stabilized to 8.3 ± 0.2 after aeration for 20 min before adding the *Lemna minor* fronds. The test samples were then incubated at 24 ± 2 °C under white fluorescent lighting for 7 days, and the frond numbers were counted at the end of the tests (i.e., on the 7th day). All tests, including the control, were repeated five times. In all control replications the plants were healthy with a bright green colour, while the number of fronds doubled in 2.1 days and had an 8-fold increase in 7 days, at the end of the

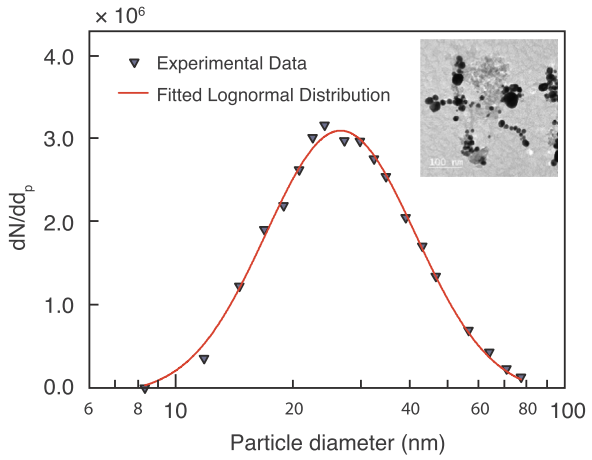

Fig. 2. Size distribution of the test AgNPs measured by the apparatus shown in route 1 of Fig. 1. Inset: Scanning Electron Microsope images of particles collected on a microscopy grid.

experiment.

To evaluate the potential toxic effect of the tested materials we estimated the Inhibition of Growth Rate (IGR) based on the measurements of the frond number, according to:

$$\mathrm{IGR} = \frac{(\mu_c - \mu_r)}{\mu_c} \times 100, \tag{1}$$

where μ_c and μ_r are the mean number of fronds in the control experiment and in the experiments with the AgNPs, respectively (ISO 20079, 2005; OECD 221, 2006). One-way ANOVA with Tukey post-hoc test ($p < 0.001$) was used to determine whether difference in the numbers of fronds measured among the various samples were significantly different or not. Half maximal effective concentration (EC_{50}) values were also determined based on the estimated IGRs according to the OECD 221 (2006) guidelines.

3. Results and discussion

Fig. 3 shows the number of *Lemna minor* fronds that grew over the period of 7 days when exposed to different concentrations of AgNPs. Compared to the control, a decrease of the total frond number by ca. 40% was observed when using AgNP concentrations as low as 5 μg L^{-1}, and by 91% when the AgNP concentration increased to 40 μg L^{-1}. For concentrations higher than 5 μg L^{-1} we also observed that the fronds had only one leaf while their colour turned from green to yellow or white, providing an additional

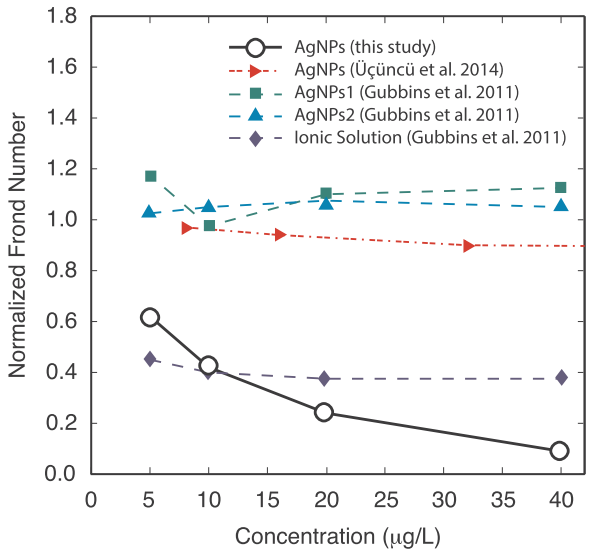

Fig. 3. Reduction of *Lemna minor* frond number when the toxicity tests are conducted with AgNPs produced by spark ablation in the gas phase (this study), AgNPs produced by classical wet-chemistry methods (Gubbins et al., 2011), AgNPs produced by laser ablation in the liquid phase (Üçüncü et al., 2014), or Ag ionic solutions (Gubbins et al., 2011).

qualitative indication of toxicity. Differences in frond numbers between all our samples on the 7th day of the tests were statistically significant (1-way ANOVA, $p < 0.001$).

The reductions in frond numbers observed in our tests are significantly higher compared to those reported by Gubbins et al. (2011) and Üçüncü et al. (2014), who also tested the toxicity of AgNPs on *Lemna minor* (their data are also included in Fig. 3). It should be pointed out, however, that Gubbins et al. used classical wet-chemistry methods to produce AgNPs having diameters from 7 to 30 nm (AgNPs1) or from 40 to 110 nm (AgNPs2), while Üçüncü et al. (2014) employed laser ablation in the liquid phase to synthesize particles with diameters from 5 to 20 nm. We should also note here that our observations are in better agreement with the measurements reported by Gubbins et al. (2011) when they used ionic silver solution. This indicates that the AgNPs we produce by spark ablation in the gas phase, dissolve to produce silver ions when inserted to the test solution, with their dissolution rate being proportional to their surface concentration as indicated by Kourmouli et al. (2018). In contrast, AgNPs generated by wet-chemistry methods such as the one used by Gubbins et al. (2011), typically have organic coatings (capping agents) that inhibit the dissolution of silver to the medium, and therefore ionic toxicity is not observed. It is not surprising therefore that those AgNPs induce a toxic effect on *Lemna minor* only at higher concentrations, as reported by Gubbins et al. (2011).

The measurements reported by Üçüncü et al. (2014) showed that AgNPs can induce a frond number reduction at concentrations as low as 8 $\mu g\,L^{-1}$. Although this threshold concentration is significantly lower compared to that observed by Gubbins et al. (2011), the relative reduction of frond numbers was considerably smaller compared to that observed in our measurements (cf. Fig. 3). This deviation can also be attributed to the difference in the level of impurities of the particles used in the two studies. Considering that Üçüncü et al. (2014) used laser ablation in the liquid phase, the level of particle impurities is higher compared to that of gas phase methods (such as spark ablation that is used here), but lower compared to those of classical wet-chemistry techniques used by Gubbins et al. (2011).

Fig. 4 shows the estimated IGRs and Table 1 the associated EC_{50} values. Estimations of IGRs and EC_{50} values from the measurements reported by Gubbins et al. (2011) and Üçüncü et al. (2014) are also shown in Fig. 4 and Table 1. The estimated EC_{50} value from our study is 8.9 $\mu g\,L^{-1}$ ($p < 0.003$), which is significantly lower compared to those reported by Gubbins et al. (i.e., 140 and 125 $\mu g\,L^{-1}$ for AgNP1 and AgNP2, respectively) and Üçüncü et al. (i.e., 25 $\mu g\,L^{-1}$), but higher compared to that of the measurements with ionic silver (ca. < 5 $\mu g\,L^{-1}$) provided from Gubbins et al. (2011).

The AgNPs of high purity used in our tests, inhibit the growth of *Lemna minor* more effectively than those produced in the liquid phase. The IGRs estimated using the measurements reported by Gubbins et al. (2011) are close to zero when the concentration of AgNPs ranged from 5 to 40 $\mu g\,L^{-1}$. In contrast, the IGRs estimated by our tests increase from ca. 40 to 90% as the concentration of AgNPs increases from 5 to 40 $\mu g\,L^{-1}$. These high IGRs can also be explained by the dissolution of AgNPs to toxic Ag^{+} ions upon insertion in the aqueous test medium due to the absence of capping agents on their surface. This hypothesis is supported by the agreement, which is within experimental uncertainty, of the IGRs estimated from our tests and those using ionic silver solutions reported by Gubbins et al. (2011). The IGRs estimated in our work are also in the same range with those reported by Üçüncü et al. (2014), who used AgNPs produced by laser ablation in the liquid phase. As suggested above, those NPs should also be free of any capping agents and more pure compared to those used by Gubbins et al. (2011), which can explain the fact that the IGRs reported by Üçüncü et al. (2014) are closer to the values reported in this work.

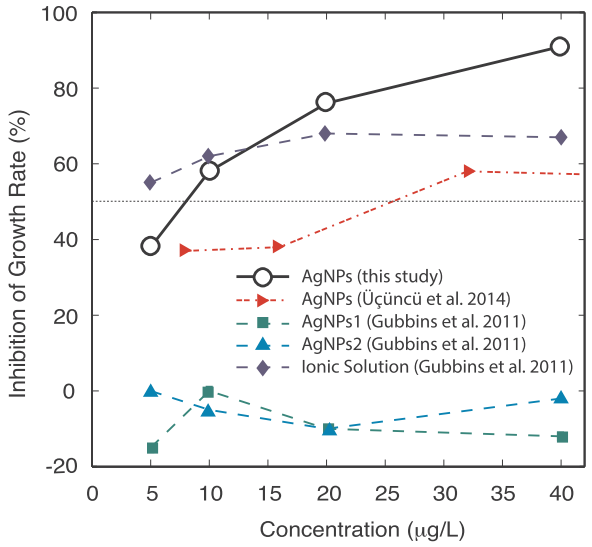

Fig. 4. Inhibition of Growth Rate (%) of *Lemna minor* frond number when exposed to AgNPs produced by spark ablation (black line with black circles; this study), AgNPs produced by classical wet-chemistry methods (green and red lines with diamonds and triangles corresponding to different particle sizes; Gubbins et al., 2011) AgNPs produced by laser ablation in the liquid phase (red line with triangles; Üçüncü et al., 2014), and ionic silver (blue line with diamonds; Gubbins et al., 2011).

Table 1
EC_{50} values ($\mu g\,L^{-1}$) for AgNPs (this study), AgNP1, AgNP2, ionic silver (Gubbins et al., 2011), and AgNPs (Üçüncü et al., 2014) based on frond number.

	This study	Gubbins et al. (2011)			Üçüncü et al. (2014)
	AgNPs	AgNP1	AgNP2	Ionic silver	AgNPs
EC_{50}	8.9	140.5	125.5	< 5	26.1

4. Conclusions

We have shown that high-purity AgNPs generated by spark ablation have a toxic effect on *Lemna minor* at concentrations as low as $5\,\mu g\,L^{-1}$. This is lower compared to the values reported by Gubbins et al. (2011), who used AgNPs generated by classical wet-chemistry techniques, and Üçüncü et al. (2014) who used AgNPs generated by laser ablation in the liquid phase. The NPs synthesized for the needs of the tests in this work are free of any capping agents and have a higher purity compared to those employed in the earlier studies. As a result, they can more efficiently release Ag^+ ions upon dissolution in aqueous solutions, which in turn can explain their higher toxicity as reflected by the lower frond number and high Inhibition of Growth Rates we observed.

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