Combined effects of silver nanoparticles and humic and fulvic acids on *Vibrio splendidus* growth

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Introduction

Over the last decade, industrial production and commercial use of engineered nanomaterials (ENMs), such as silver nanoparticles (AgNPs) and carbon nanotubes (CNTs), have significantly increased worldwide. Due to their antimicrobial properties, silver nanoparticles (AgNPs) are now extensively used in many consumer-products[1] like anti-odor textiles and food packaging[2] as well as in many processes such as[1], water purification and production of antifouling and aseptic surfaces.[3] ENMs can enter aquatic environments from their manufacturing processes to their disposal. As a consequence, the USEPA has included them among the emerging aquatic contaminants since 2009.[4] Nevertheless, potential toxic effects of these emerging contaminants on natural aquatic communities, from bacterial communities to vertebrates, are still not well understood.[5] Many studies have described the effects of AgNPs and ionic silver on pathogen microorganism models such as bacterial species of the genus *Vibrio*, *Escherichia* and *Pseudomonas*[6–9] as well as on complex bacterial communities,[10–12] but few on environmental bacteria. Moreover, only few environmental studies are taking into account the fact that, in natural aquatic environments, the occurrence of natural organic matter (NOM) could interact with AgNPs[13] and, as a consequence, modify their toxicity against aquatic organisms[14,15] and microorganisms.[16] These studies have demonstrated that humic acids (HA) and fulvic acids (FA), two important components of the ubiquitous NOM,[17] could interact with AgNPs.[13,18–20] The adsorption of FA, HA or NOM onto nanoparticles enhances electrostatic or steric stability. Aggregation of AgNPs could be modified by this new coating at two different levels, at low ionic strength NOM decreases AgNPs aggregation rates, moreover in presence of divalent cation, aggregation processes are promoted by NOM addition.[21] AgNPs stability influences their toxicity for microorganisms. In estuarine waters, Millour *et al.*[22] have recently observed a rapid change in the size of AgNPs influenced by NOM concentration, nevertheless this aggregation was not observed in nanopure water. As a consequence, to better understand the ecotoxicological risk of AgNPs in aquatic environments it is essential to characterize their biocidal (toxicological) effects in association with HA and FA towards bacteria inhabiting these ecosystems. The aim of this study was to determine the toxicity of AgNPs on *Vibrio splendidus* growth, a well-known bacterial pathogen, with and without the addition of two NOM components, HA and FA.

Materials and Methods

Bacterial culture conditions

Vibrio splendidus 7SHRW,[23] an environmental strain isolated from the Gulf of St. Lawrence (Quebec, Canada), was grown overnight in LB medium at room temperature. This strain has been previously used in our laboratory and was chosen due to its sensibility to ENMs.[24] Cultures were centrifuged at 4000 rpm for 5 min. *Vibrio* cell pellets were washed twice in physiological water (9‰ of NaCl, pH 7.2, 0.2 µm filtered and autoclaved) and finally in nanopure water (NW, pH 7.2, 0.2 µm filtered and autoclaved) to remove any residual growth medium. Then, *Vibrio* cell suspensions were diluted in sterile NW to obtain working bacterial suspensions containing 10^7 cell.mL^{-1}.

AgNPs, HA and FA exposition conditions

The different xenobiotics used in this study were AgNPs-citrate 20 nm (synthesised at E. Pelletier's laboratory-ISMER), Suwannee River humic and fulvic acids standard II (IHSS, St. Paul, MN, USA). At first, the effects of a single exposition of *Vibrio* cells to AgNPs (0, 20, 100 and 1000 µg.L^{-1}), HA and FA (0, 2.5, 10 and 50 mg.L^{-1}) were assessed. Then, the effects of different combinations of AgNPs/HA and AgNPs/FA, at the same concentrations than previously used during single expositions, were investigated. Bacterial cell expositions to silver nanoparticles were performed in triplicates in nanopure water into 1.5 mL centrifuge tubes. The nanopure water avoids the risk of agglomeration of AgNPs. Tubes were kept rotating on a shaker incubator at 100 rpm for 2 h at ambient temperature. After exposition, 100 µL of each mix were transferred into 96-well plates containing 100 µL of 2X LB medium. Cell growth was estimated by measuring the optical density (OD) at 595 nm during 72 h on a Multiskan Ascent Microplate Photometer (Thermo Scientific). The growth curves were obtained by plotting OD values *versus* growth time. The specific growth rate (µ) of each treatment was estimated from the slope regression of ln ($OD_{595\ nm}$) *versus* exponential growth time.[25]

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Statistical analyses

All statistical analyses were done using SYSTAT software version 12.0 (Systat Software Inc., Chicago, USA) with α=0.05. Data normality was examined using the Kolmogorov–Smirnov. Differences between treatments were tested using a 1-way ANOVA. For subsequent multiple comparisons, Tukey's tests was performed when appropriate.

Results and Discussion

Single exposition to AgNPs, HA or FA

On a global scale, the growth rate and the lag time required before exponential growth phase of *V. splendidus* cells were significantly affected by a 2 h exposition to AgNPs at 100, 500 and 1000 µg.L^{-1} ($P<0.001$), HA at 10 and 50 mg.L^{-1} ($P<0.001$) and FA at 10 mg.L^{-1} ($P<0.001$) (Table 1).

At low concentration (20 µg.L^{-1}), AgNPs did not influence the growth rate of *V. splendidus* cells. At medium concentration (100 µg.L^{-1}), cell growth rate and lag time increased from 9 to 20 h ($P<0.001$) (Table 1). At high AgNPs concentration (1000 µg.L^{-1}), no bacterial growth was observed during the time of the experimentation. Hence the biocidal activity of AgNPs alone is between 100 and 1000 µg.L^{-1} (geometric mean of 316 µg.L^{-1} = threshold

effect). Increasing of the lag time has been already observed on other bacterial strains exposed to AgNPs.[3,16] The extension of the lag time suggests that bacterial cells were able to adapt to the presence of AgNPs at low-medium concentrations, whereas at higher concentrations, AgNPs are bactericidal for bacterial cells.

The growth rate of *V. splendidus* cells was not affected by addition of HA up to 10 mg.L^{-1}. However, at 50 mg.L^{-1}, HA demonstrated a bactericidal effect (Table 1). During FA expositions, all concentrations significantly decreased the bacterial growth rate and increased the lag time (Table 1). Even if FA effects are concentration-dependant, no bactericidal effect was observed at the highest concentration tested. This result differs from the results obtained during HA expositions. The different toxicity of HA and FA, two components of NOM, toward *Vibrio splendidus cells* could be related to their structural differences (molecular weight, amount of functional groups, atomic composition[20]) which can modify their interactions with the cells. Even if HA effects on biological cells are relatively well documented,[18] only few information are actually available concerning FA. Moura *et al.*[19] have demonstrated the existence of a rapid sorption of HA and FA on *Bacillus subtilis* cell surfaces. This sorption may affect structure, fluidity and permeability of bacterial cell membranes.[26] In addition, humic substances could be internalized by bacterial cell[27] and used as carbon source.[18] As a consequence, the HA/FA ratio in surface waters could dictate the toxic outcome of AgNPs.

Table 1. Effects of the different concentrations of AgNPs, HA and FA on growth rate and on lag time of the *Vibrio splendidus*.

Treatments	Growth rate (h^{-1})	Lag time (h)
Control	0.13±0.00	9
AgNPs 20 µg.L^{-1}	0.13±0.00	11
AgNPs 100 µg.L^{-1}	0.18±0.01	20
AgNPs 1000 µg.L^{-1}	*	*
HA 2.5 mg.L^{-1}	0.15±0.05	12
HA 10 mg.L^{-1}	0.08±0.01	24
HA 50 mg.L^{-1}	*	*
FA 2.5 mg.L^{-1}	0.11±0.00	12
FA 10 mg.L^{-1}	0.08±0.00	14
FA 50 mg.L^{-1}	0.10±0.00	33

*No bacterial growth observed during the time of the experimentation.

Table 2. Combined effects of the different concentrations of the AgNPs, HA and FA on growth rate and on lag time of the *Vibrio splendidus*.

	Growth rate(h^{-1})					
	HA 2.5 mg.L^{-1}	HA 10 mg.L^{-1}	HA 50 mg.L^{-1}	FA 2.5 mg.L^{-1}	FA 10 mg.L^{-1}	FA 50 mg.L^{-1}
AgNPs 20 µg.L^{-1}	0.10±0.01	0.13±0.00	0.11±0.00	0.10±0.00	0.12±0.00	0.10±0.00
AgNPs 100 µg.L^{-1}	0.15±0.01	0.22±0.00	0.11±0.00	0.13±0.00	0.20±0.00	0.11±0.00
AgNPs 1000 µg.L^{-1}	0.08±0.00	0.08±0.00	0.11±0.00	0.10±0.00	0.11±0.00	0.10±0.00
	Lag time(h)					
	HA 2.5 mg.L^{-1}	HA 10 mg.L^{-1}	HA 50 mg.L^{-1}	FA 2.5 mg.L^{-1}	FA 10 mg.L^{-1}	FA 50 mg.L^{-1}
AgNPs 20 µg.L^{-1}	24	26	33	24	25	33
AgNPs 100 µg.L^{-1}	16	22	33	15	21	33
AgNPs 1000 µg.L^{-1}	32	32	33	32	33	33

Toxicity of AgNPs/HA and AgNPs/FA combinations

Single expositions to each xenobiotic (AgNPs, HA and FA) are essential to better understand the specific impacts on bacterial cell growth. Nevertheless, when considering the release of AgNPs in complex natural media, such as aquatic environments, it is essential to consider the effects of xenobiotic interactions on bacterial cells to adequately estimate the ecotoxicological risk of these nanomaterials. Our results clearly demonstrated that combined expositions of bacterial cells to AgNPs/HA and to AgNPs/FA significantly modified the growth rate of *V. splendidus* (Table 2). No significant difference could be observed in bacterial growth rate when the cells were exposed at 20 µg.L^{-1} AgNPs whatever the concentrations of HA or FA added in the medium (Table 2). Nevertheless, the lag time increased with increasing NOM concentration (Table 2). At 100 µg.L^{-1} AgNPs, the addition of 10 mg.L^{-1} FA (or HA) increased the growth rate and lowered lag time compare to the control without NOM (Table 2), suggesting a reduction of the AgNPs toxicity by HA and FA. Contrary to the results obtained during single expositions, the combination of the highest AgNPs and HA concentrations allowed bacterial growth after approximately 33 h (Table 2). These observations are consistent with Su *et al.*[28] who demonstrated that antibacterial effect of carbon nanotubes decreased in presence of 10 mg.L^{-1} HA due to the adsorption of HA on the nanotube surface. The reduction of AgNPs toxicity at 1 mg.L^{-1} had also been observed on bacterial cells in biofilms by the addition of 10 mg.L^{-1} HA.[13] AgNPs are supposed to interact mainly with bacteria through their dissolution into Ag^+ ions. As a consequence, reduction of AgNPs toxicity could be due to a complexation of HA with Ag^+ or to a coating of AgNPs by HA that could prevent the release of Ag^+ ions. Liu and Hurt[29] have demonstrated that NOM addition reduces the dissolution of AgNPs in water in a dose dependent manner. This could explain the reduction of AgNPs toxicity towards *Vibrio splendidus* cells observed at the highest concentrations used in our study. The effect of HA addition is higher than FA addition. As previously mentioned, HA and FA have a different chemical composition and could interfere differentially with AgNPs, and as a consequence with the liberation of Ag^+ ions.

Conclusions

In conclusion, our results demonstrated that the toxicological properties of AgNPs toward *Vibrio splendidus* cells are modified by the addition of HA and FA. As a consequence, risk assessment of nanomaterials in aquatic ecosystems requires to take into account the chemical (salts) composition of waters where AgNPs are dispersed but also the co-occurrence of NOM and their chemical composition (HA or FA) that could modify nanomaterials-xenobiotics toxicology.

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