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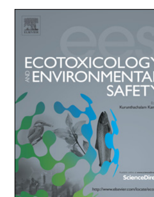

Qualitative toxicity assessment of silver nanoparticles on the fresh water bacterial isolates and consortium at low level of exposure concentration

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ABSTRACT

Silver nanoparticles (AgNPs) pose a high risk of exposure to the natural environment owing to their extensive usage in various consumer products. In the present study we attempted to understand the harmful effect of AgNPs at environmentally relevant low concentration levels (≤ 1 ppm) towards two different freshwater bacterial isolates and their consortium. The standard plate count assay suggested that the AgNPs were toxic towards the fresh water bacterial isolates as well as the consortium, though toxicity was significantly reduced for the cells in the consortium. The oxidative stress assessment and membrane permeability studies corroborated with the toxicity data. The detailed electron microscopic studies suggested the cell degrading potential of the AgNPs, and the FT-IR studies confirmed the involvement of the surface groups in the toxic effects. No significant ion leaching from the AgNPs was observed at the applied concentration levels signifying the dominant role of the particle size, and size distribution in bacterial toxicity. The reduced toxicity for the cells in the consortium than the individual isolates has major significance in further studies on the ecotoxicity of the AgNPs.
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1. Introduction

Silver nanoparticles (AgNPs) are being extensively used in consumer products like cosmetics, soaps, paints, and optical devices (Fabrega et al., 2011). The AgNPsalso have extensive usage in home, medical appliances and in various food industries as preservatives (Konopka et al., 2009; Silvestry-Rodriguez et al., 2008). According to the Woodrow Wilson Inventory (2013) silver nanoparticles (AgNPs) are the dominating nanomaterials in the consumer products. The silver nanoparticles released directly or indirectly by the different industries and the wastewater treatment plants may ultimately end up in the aquatic environment (Ju-Nam and Lead, 2008). Owing to the steep increase in the usage and production of AgNPs in recent times, it is pertinent to study the possible risk associated with the exposure of AgNPs and its detrimental effect on the environment.

Bacteria are considered to be among the sensitive species towards the toxic effects of the engineered nanomaterials. However, depending on the tested bacterial species, biotest system or the specific particle type, the toxicity values of Ag may vary from nanogram Ag/L to milligram Ag/L (Fabrega et al., 2011; Marambio-Jones and Hoek, 2010). Although the AgNPs are well known for their antibacterial effects the mechanism for the toxic effect of AgNPs is still unclear. AgNPs can attach to the surface of the cell membrane and disturb the permeability and respiratory functions of the cell (Kvitek et al., 2008). They can also penetrate the bacterium and cause further damage (Morones et al., 2005). The possible toxic impacts of the AgNPs towards the freshwater bacterial species may reflect the possible interference that could impose substantial damage to the other members of the microbial communities in the aquatic food web. Additionally, there have been alarming reports regarding the possible transfer of these nanoparticles from the prey (like *Pseudomonas aeruginosa*) to the predator (like *Tetrahymena thermophila*) in a typical aquatic ecosystem (Kessler, 2011).

Though there are extensive reports on the possible effects of AgNPs on the individual bacteria sp. (Sharma et al., 2009), a thorough literature survey reveals that the possible impact of the AgNPs on a mixed bacterial culture has not been explored so far. Second, the prior studies were mostly carried out with AgNPs in a defined growth media, where the medium components could affect the stability of the AgNPs and influence their toxic effects (Cumberland and Lead, 2009). Our previous study on Al_2O_3 NPs using lake water matrix (Pakrashi et al., 2011) suggested that NP

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stability played a key role in assessing ecotoxicity. Finally, based on the current production rate the 'environmental' concentrations of the AgNPs have been modelled to be below 1 μg/mL (Gottschalk et al., 2009). Hence, the present study deals with possible toxic effects of AgNPs at environmentally relevant low concentrations ($\leq$1 ppm) towards two different freshwater bacterial isolates and their consortium in the sterile lake water medium without any nutrient supplements to mimic the chemical matrix of freshwater aquatic environment. Standard plate count assay was done to study the bacterial viability before and after the NP interaction. Oxidative stress and membrane permeability assessment were carried out to determine the possible mechanism of AgNPs toxicity. The possible involvement of surface functional groups in the cell–NP interaction was assessed through FT-IR analysis. Optical and electron microscopic studies were carried out to observe the cell damaging potential of the AgNPs. The possible involvement of Ag^+ ions in cytotoxicity was also studied.

2. Materials and Methods

2.1. Materials

Silver nano powder (Ag 5 percent trace metals PVP as dispersant, particle size <100 nm CAS No. 57683299) and DCFH-DA (2′,7′-dichloroflouroscence diacetate) were purchased from sigma Aldrich (St. Louis, MO, USA). Acridine Orange (AO) and Ethidium bromide (EtBr) were obtained from Hi-Media Pvt. Ltd., (Mumbai, India). Fresh water was collected from the VIT lake Vellore, India (12°58′10″N, 79°9′37″E). All the chemicals used in the experiments were of analytical grade.

2.2. Characterization of silver nanoparticles (AgNPs)

2.2.1. X-ray diffraction (XRD)

To characterize the procured AgNPs, X-ray diffraction analysis was carried out (Model D8, Advanced X-ray Diffractometer, Germany) using Cu-$K\alpha 1$ wavelength (=1.54059 Å), scanning in 2θ range from 20° to 80°. AgNPs powder was grinded to a fine powder, and a uniform smear was prepared on a glass slide. The prepared sample was packed into a sample container for analysis. The crystalline structure of the AgNPs was determined with the diffraction patterns obtained.

2.2.2. Transmission electron microscopy (TEM)

Particle morphology of the as-received AgNPs was studied with the help of transmission electron microscopy (Philips CM12, The Netherlands). The AgNPs powder was dispersed in Milli-Q water [Pall—corporation, Cascade biowater] using ultrasonicator (Sonics., USA) for 10 min at a concentration 10 μg/mL to achieve uniform dispersion of the NPs. The sample was aerated and then placed onto lacey —carbon coated copper grid for transmission electron microscopy analysis.

2.3. Stability analysis of nanoparticles dispersion

A stock dispersion of AgNPs (100 μg/mL) was prepared in Milli-Q filtered water and sonicated using ultrasonic processor (Sonics., USA) of 750 W for 10 min. A working concentration of 1 μg/mL was prepared in filtered lake water by diluting the required volume of stock solution and the hydrodynamic size of the particles in the suspension was measured at time intervals of 0 h, 2 h, 4 h, and 6 h by Dynamic light scattering method using a 90 plus Particle Size Analyzer(Brookhaven Instruments Corporations, USA).

AgNP dispersions (1 μg/mL) in the filtered lake water were incubated for 2 h, 4 h and 6 h in a shaker at room temperature. After the stipulated time intervals, the dispersion was centrifuged three times at 12,000 rpm at 4 °C for 20 min, and then subsequently filtered through 0.1 μm and 10 kDa membrane filters as discussed in our previous reports (Pakrashi et al., 2011; Dalai et al., 2012). The complete removal of AgNPs was ensured by carrying out the hydrodynamic size analysis of the filtrates (Pakrashi et al., 2011; Dalai et al., 2012). The concentration of Ag+ ions in the suspension was measured using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Perkin Elmer Optima 5300 DV, USA) with a detection limit of 1 μg/L. Before the analysis, samples were acidified using 0.1 N HNO_3 to ensure availability of soluble Ag bound to organic substances present in lake water.

The actual concentration of the AgNPs in the medium was measured by atomic absorption spectroscopy (AAS) at a wavelength of 338.29 nm using a flame atomic absorption spectrophotometer (Analyst400/HGA 900, PerkinElmer, USA). Four different concentrations, i.e. 0.25 μg/mL, 0.50 μg/mL, 0.75 μg/mL and 1 μg/mL were prepared by adding desired volume of stock in 20 mL working volume of lake water. The samples were then acid digested and analyzed by AAS.

2.4. Isolation and identification of test organisms

The water was collected from three different sites VIT Lake and stored in polypropylene bottles at 4 °C. Serial dilution of the sample was carried out in sterile distilled water. The diluted sample was spread to plate containing nutrient agar (Himedia, Catlog No. GRM666-Agar Agar and Himedia Catlog No. M002-Nutrient Broth)and incubated for 24 h at 37 °C. The incubation was done at 37 °C since this was the optimum temperature for the bacterial growth, and also the temperature of the VIT Lake was noted to be in the range of 35 ± 5 °C. After 24 h, the observed bacterial colonies with different colony morphology were isolated and labelled. The different isolates were cultured on nutrient agar medium to obtain pure cultures. The morphological observations, biochemical tests and Gram staining were performed to ensure the genus level difference between the isolates. The two dominant bacterial species in the lake water, identified as *Bacillus thuringiensis* and *Bacillus aquimaris*, were named as *B. thuringiensis* VITLW1, and *B. aquimaris* VITLW2. The isolated lake water bacteria showed a 99 percent similarity in BLAST search to the available corresponding sequences.

2.5. Characterization of the selected isolate

To confirm the identity of selected isolates 16S rRNA sequencing was performed. Phenol–chloroform method (Sambrook et al., 1989) was employed for genomic DNA isolation from the bacterial strain grown in NB medium. The 16S rRNA nucleotide sequencing was performed by fluorescent dye terminator method (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). The sequence obtained was analysed using BLAST. The nearest neighbouring sequences were downloaded and aligned using ClustalW version 1.6. Phylogenetic tree was constructed using aligned sequences by the neighbour joining algorithm using CLC free workbench 3.2 software. On the basis of different morphology, biochemical characteristics and 16S rRNA gene analysis; the taxonomic identity of the strain was confirmed.

2.6. Development of binary consortium

To analyze the toxicity of AgNPsa bacterial consortium (a mixture of cultures) involving two individual bacterial species was prepared. For developing the consortium, antagonistic and synergistic studies was carried out with two bacterial isolates (*B. thuringiensis*, *B. aquimaris*). The isolate *B. thuringiensis* was taken and grown in nutrient broth, after 4 h of incubation; 100 μL of culture broth was poured onto the surface of nutrient agar and a loop full culture of the isolate *B. aquimaris* was streaked in the middle of the plate. It was incubated at 37 °C for 24 h, and then the plate was observed. The absence of the zone of inhibition between the two isolates confirmed that they lacked competitive inhibition (Samuel et al., 2012a). In the subsequent sections "consortium" essentially means "binary consortium of *B. thuringiensis,* and *B. aquimaris*".

2.7. Toxicity assessment

2.7.1. Experimental set up

The lake water was collected from VIT lake, Vellore, India. The study has been carried out in a laboratory away from the lake. For toxicity assessment we have employed typical experimental set up as detailed in our previous publications on ecotoxicity assessment of Alumina, and Titania nanoparticles on freshwater bacteria (Pakrashi et al., 2011; Dalai et al., 2012). Briefly, a stock dispersion of AgNPs (100 μg/mL) was prepared in Milli-Q water and subjected to ultrasonication at 750 W for 15 min (Sonics, USA). The test NP suspension of 0.25 μg/mL, 0.50 μg/mL, 0.75 μg/mL, 1 μg/mL final concentration was prepared by diluting the required volume of stock dispersion. Physical parameters of the freshwater were measured as follows: conductance: 4.5 ± 0.17 mS/cm; pH: 7.7 ± 0.2; dissolved oxygen (DO): 7.4 ± 0.49 mg/L; total dissolved solids (TDS): 820 ± 80 mg/L; total organic carbon (TOC): 15 mg/C/L. The freshwater was filtered through Whatman no. 1 filter paper and to ensure the absence of larger colloids and then autoclaved then autoclaved to remove natural bacterial community. The filtrate was used as the test media throughout this study.

All the experiments were carried out on the bacterial species and the consortium developed with the help of the two isolates *B. thuringiensis* and *B. aquimaris*. The bacterial cells were first inoculated in the nutrient broth and in the exponential phase the cells were harvested by centrifugation at 7000*g* for 10 min. Lastly the cells were washed twice with the sterilised lake water to remove growth media components. From standard plate count assay initial bacterial cell number was found to be 215×10^7. Since the experiments were to be carried out under nutrient deficient conditions, the cell number was kept sufficiently high to facilitate toxicity assessment following our previous publications (Pakrashi et al., 2011; Dalai et al., 2012). Due to overgrowth of numerous bacterial colonies (>300 CFU/mL) in the as collected Lake water i.e. in the natural condition it was difficult to count the number of colonies ruling out attempts to compare the numbers in these two cases. To maintain accuracy, all the experiments were performed in the triplicates, and the standard error was calculated. Individual bacterial isolates and the consortium

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