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Cytotoxicity of Al_2O_3 Nanoparticles at Low Exposure Levels to a Freshwater Bacterial Isolate

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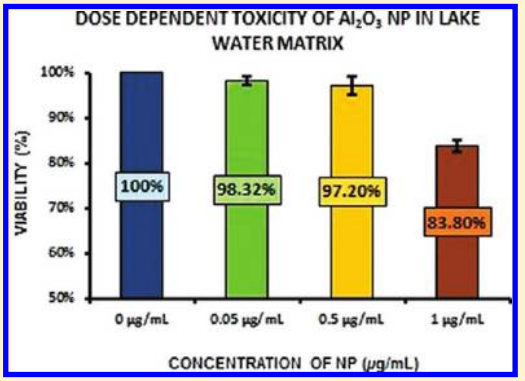
Supporting Information

ABSTRACT: The cytotoxicity of Al_2O_3 nanoparticles (NP) at very low exposure levels (1 μg/mL and less) to a dominant bacterial isolate from freshwater (lake water), *Bacillus licheniformis*, was examined. Sterile lake water was directly used as a test medium or matrix to simulate the freshwater environment. Exposure to 1 μg/mL Al_2O_3 NP for 2 h caused a 17% decrease in cell viability (as determined by plate count and MTT assay). During the test period, the particles were found to be stable against aggregation in the matrix and exerted a nano-size effect on the exposed test organisms. The decrease in cell viability was proven not to be due to the release of Al^{3+} ions from the nanoparticles in the dispersion. The zeta potential and FT-IR analyses suggested that the surface charge based attachment of nanoparticles on to the bacterial cell wall was responsible for flocculation leading to toxicity. The cell wall damage confirmed through SEM and the lipid peroxidation assay also contributed toward toxicity. This study warns of possible ecotoxicity of nanoparticles even at environmentally relevant concentrations. However, detailed studies need to be carried out to establish probable mechanistic aspects of this low concentration toxicity phenomenon.



INTRODUCTION

With rising industrial applications of engineered nanoparticles, they are destined to enter the aquatic, terrestrial, and atmospheric zones in the environment, where their fate and behavior are yet to be determined.[1] The unique properties and small size of the engineered Al_2O_3 NP have inevitably increased their application potential. Al_2O_3 NP has a wide range of applications as coatings and abrasives and as additives in the fields of composites and heat transfer-enhancing nanofluids.[2,3] The influence of Al_2O_3 NP on blended concrete, in terms of its compressive strength, has recently been explored.[4] Engineered nanoparticles, owing to their extensive commercial applications, have received considerable attention regarding possible environmental impacts.

The fate, behavior, and transportation of nanoparticles in different water matrixes (groundwater, surface water, and seawater) are poorly understood, as is their toxicity potential. Mostly, this differential behavior depends on the nanoparticle size and stability in the dispersion medium.[5] The potential release of these nanoparticles in the environment has necessitated an understanding of their stability, mobility, and reactivity.[1] A recent study showed that the behavior and stability of oxide nanoparticles can differ significantly depending on the test medium.[6]

Exposure to nanoparticles could cause oxidative stress in cells at the cellular and subcellular levels and also releases toxic constituents (i.e., secondary toxicity).[7] The toxicity of nanoscaled aluminum, silicon, titanium, and zinc oxide to different bacteria were studied in the recent past.[8] Al_2O_3 NP demonstrated a mortality rate of 57% to *Bacillus subtilis*, 36% to *Escherichia coli*, and 70% to *Pseudomonas fluorescens* at a low concentration (20 μg/mL) level using 1 g/L NaCl as the experimental medium. In a nutrient enriched test medium, we studied the antimicrobial sensitivity of Al_2O_3 NP to *E. coli* and illustrated a nominal growth inhibitory effect of NP only at very high concentrations (>1000 μg/mL).[9] Another study by our group demonstrated the growth inhibitory effect of Al_2O_3 NP on *Scenedesmus* sp. and *Chlorella* sp. (72 h EC_{50} 39 μg/mL and 45 μg/mL, respectively).[10] A study on yeast cells demonstrated the membrane disruption property of SiO_2 and Al_2O_3 NP at high concentrations (>1000 μg/mL), whereas at same concentrations, HfO_2 and CeO_2 did not show any significant effect.[11] A study focused on the mechanistic aspects of TiO_2 and Al_2O_3 NP toxicity suggested the possibilities of non-ROS mediated toxicity toward certain invertebrate systems.[12] In another assessment, toxicity and bioaccumulation of Al_2O_3 NP to a range of sediment dwelling microorganisms (such as *Tubifex tubifex*, *Hyalella azteca*, *Lumbriculus variegatus*, and *Corbicula fluminea*) were observed. Toxicity was found to be more in nano-Al_2O_3 as compared to micrometer-sized Al_2O_3 toward the survival of *H. azteca* in a 14-day exposure period upon direct contact with a thin layer of 625 mg or 2500 mg of Al_2O_3 NP.[13] Experiments on *Daphnia magna* with nano-Al_2O_3 reported 48 h EC_{50} and LC_{50} values of 114 and 162 μg/mL, respectively.[14]

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Unicellular organisms like bacteria and/or algae are assumed to be more resistant to the toxic effects of NP than protists and metazoans, which possess highly developed systems for the internalization of nano and microscale particles.[15] The toxicity of NP toward the bacterial species in the aquatic environment reflects the possible agitation that could impose damage to other members of the microbial communities in the biogeochemical cycles. There are also alarming reports regarding the transfer of engineered nanomaterials from prey (like *Pseudomonas aeruginosa*) to predator (like *Tetrahymena thermophila*) in a typical aquatic ecosystem.[16] A recent study showed that the gold nanorods readily passed from water column to the marine food web in three laboratory-constructed estuarine mesocosms containing seawater, sediment, sea grass, microbes, biofilms, snails, clams, shrimp, and fishes.[17]

As the prior reports revealed that most of the ecotoxicity tests conducted with nanomaterials had two key limitations, (i) they were undertaken in defined growth media. In contrast, we have employed sterile lake water without nutrient supplements to simulate the chemical matrix of the fresh water aquatic environment. (ii) The tested concentration ranges were kept high enough to demonstrate the toxicity (atleast above 10 μg/mL). However, based on the current production rate, the environmental concentrations of different nanomaterials (TiO_2, ZnO, Ag, CNT, and fullerenes) have been modeled to be below 1 μg/mL.[18] To evaluate the environmental risk of these nanoparticles, it is necessary to take up the toxicity study at a low concentration of these particles to analyze the impact (if any). This motivated us to evaluate the toxicity of Al_2O_3 NP at low concentration ranging from 0.05 μg/mL to 1 μg/mL.

In the present study, we aim to demonstrate the differential behavior of Al_2O_3 NP in the lake water matrix and its reactivity toward the dominant bacterial species within a short exposure period of 2 h.

MATERIALS AND METHODS

In this present study, surface water (collected from VIT Lake, Vellore, India) was used as a test medium with no nutrient supplements. Physicochemical characterization showed a conductance of 4.3 ± 0.13 mS/cm, pH of 7.8, dissolved oxygen content of 7.2 ± 0.46 μg/mL, and total dissolved solids content of 800 ± 74 μg/mL. The presence of metal ions in the lake water was quantified by ICP-OES (Perkin-Elmer Optima 5300 DV, USA) and other inorganic ions by the titrimetric method (Supporting Information). Primary filtration through a 20 μm sieve was done to remove the suspended solid particles. Subsequent filtrations through Whatman no.1 filter paper followed by 0.22 μm membrane filter were done to avoid the interference of large colloidal particles. The suspension thus obtained will henceforth be denoted as filtered lake water.

Characterization of Al_2O_3 NP Dispersion. Dry Al_2O_3 NP (γ-phase alumina nanopowder, particle size <50 nm) was procured from Sigma Aldrich (St. Louis, Missouri; CAS Number 1344-28-1). The size and shape of as procured Al_2O_3 NP were analyzed through transmission electron microscopy (Philips CM12 Transmission Electron Microscope, The Netherlands). The hydrodynamic size was measured using a particle size analyzer (90 plus Particle Size Analyzer, Brookhaven Instruments Corporations, USA) (Supporting Information).

The stock dispersion of Al_2O_3 NP (50 μg/mL) was prepared from filtered lake water and subjected to sonication using an ultrasonic processor (Sonics, USA). The hydrodynamic size of the particles or particle agglomerates was determined through dynamic light scattering. The stability of the particle size distribution was analyzed for a period of 48 h (data shown for 4 h) (Supporting Information). No change in pH was observed during the course of the experiment.

Cell Viability Evaluation. Dominant bacterial species from lake water was isolated, characterized, and identified using 16S rRNA analysis (Supporting Information). The initial cell population of 10^9 CFU/mL was used throughout the study.

The required volume of Al_2O_3 NP dispersion in filtered lake water was added to the bacterial culture to prepare exposure concentrations of 1 μg/mL, 0.5 μg/mL, and 0.05 μg/mL, respectively. Bacteria and NP interaction was carried out through proper mixing in an incubation shaker for 2 h (details of the procedure are in Supporting Information).

The cellular viability was determined using the MTT assay, which has been a well-reported tool for both eukaryotic and prokaryotic systems.[19,20] After 2 h of interaction, 0.5 mL of the samples from both the control and test were collected in separate vials. About 40 μL of MTT solution (5 mg dissolved in 1 mL 0.1 M PBS) was added. The vials were then subjected to gentle mixing in dark conditions at 30 °C for 4 h. After 4 h, 200 μL of DMSO was added to each tube, and gentle mixing was carried out. Two hundred microliters of each sample was then loaded to the wells of a microtiter plate. The absorbance was read at 570 nm (ELISA plate reader, Biotek, Powerwave XS2). Al_2O_3 NP did not show any interference with the MTT assay. The survival percentage was obtained by dividing the number of viable cells to the total number of cells in the control.[21] The observations were verified by the standard plate count assay and lipid peroxidation assay (Supporting Information).

The possible toxic effect of the released Al^{3+} ions from the nanoparticles during the test period (2 h) was analyzed in a separate experiment. Al_2O_3 NP dispersion (1 μg/mL) in the filtered lake water was incubated for 2 h in a shaker at room temperature. After incubation, the dispersion was subjected to centrifugation at 10000*g* for 20 min, followed by filtration through a 0.1 μm membrane filter.[22] The hydrodynamic size analysis was performed to ensure the total removal of Al_2O_3 NP. The concentration of Al^{3+} ion in the suspension was measured using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Perkin-Elmer Optima 5300 DV, USA). The obtained Al_2O_3 NP free suspension was interacted with the bacterial culture for 2 h and was further analyzed by the MTT assay and standard plate count assay.

Zeta Potential Measurement. The zeta potential of Al_2O_3 NP dispersion and bacterial samples was measured (90 Plus Particle Size Analyzer, Brookhaven Instruments Corporations, USA) at pH 7.0. Different concentrations (0.05, 0.5, and 1.0 μg/mL) of Al_2O_3 NP dispersion and bacterial suspension of 10^9 CFU/mL were prepared in filtered lake water (pH 7.0). Al_2O_3 NP dispersion and bacterial cells in the lake water were diluted with 0.1 mM KCl prior to analysis as per the requirement.

Scanning Electron Microscopic Analysis (SEM). The bacterial cultures interacted with 1 μg/mL of Al_2O_3 NP were subjected to scanning electron microscopic analysis (Model S-3400N, HITACHI). Standard protocol was followed for sample preparation (Supporting Information).

Statistical Analysis. All *in vitro* toxicity tests were carried out in triplicate, and the data are presented in figures as the mean value ± standard error. The data was processed using one-way ANOVA, followed by Dunnett's post-hoc test at $p < 0.05$ for the MTT assay and standard plate count assay. The data for the lipid peroxidation assay and ionic analysis were processed through Student's *t* test at $p < 0.05$. Statistically significant data points are denoted with the symbol #.

RESULTS AND DISCUSSION

Characterization of the Al_2O_3 Nanoparticle. The transmission electron micrograph suggested the presence of nearly spherical Al_2O_3 NP with varying diameters in the range of 10−70 nm (Figure 1A). The hydrodynamic size of the nanoparticles were found to be in two ranges, 62−65 nm and 78−88 nm diameter, respectively, with a *z*-average size of 76 ± 0.06 nm

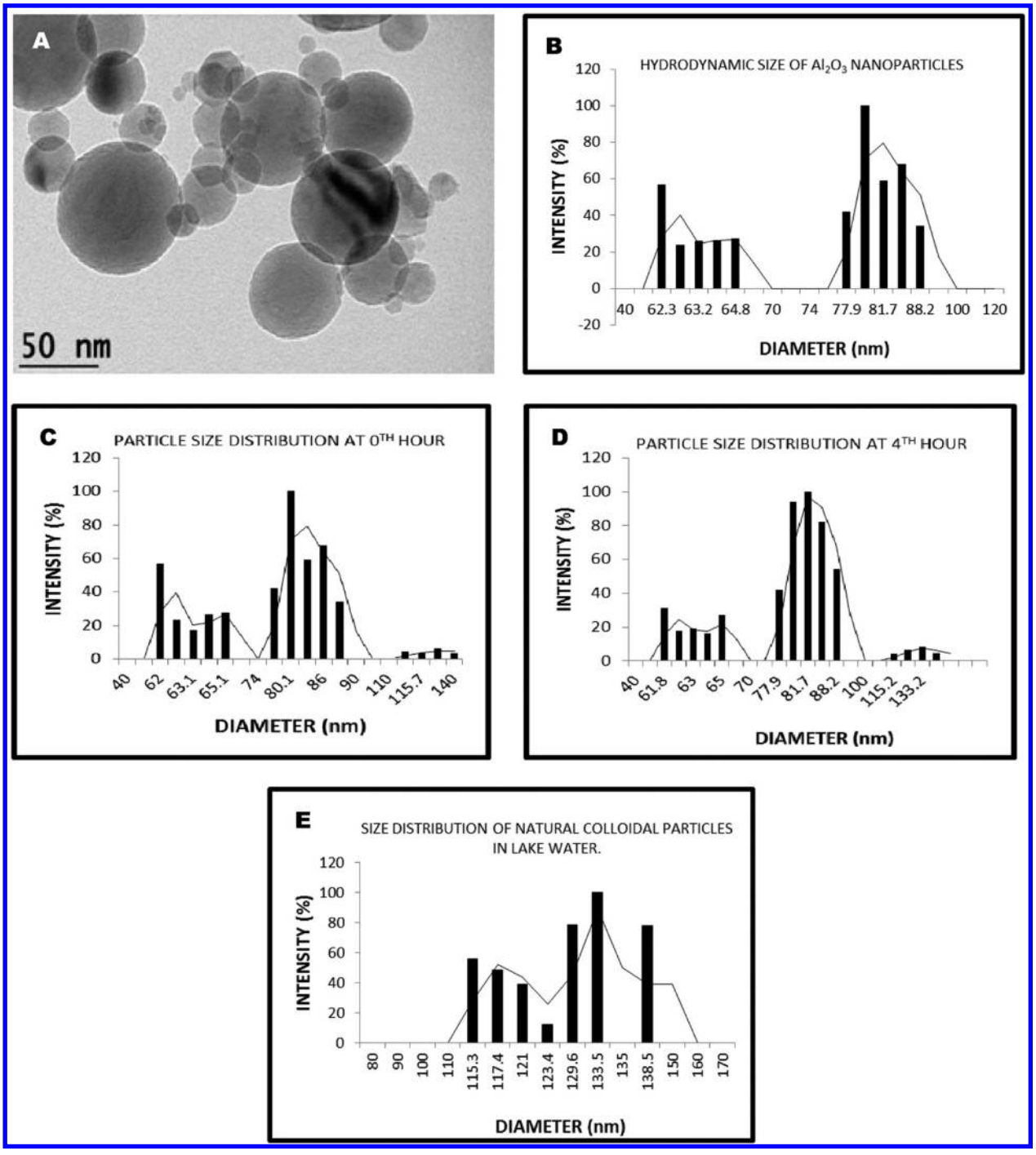

Figure 1. (A) Transmission electron micrograph of as received Al_2O_3 NP. (B) Hydrodynamic size distribution of Al_2O_3 NP measured in Millipore purified water using a dynamic light scattering particle size analyzer. (C) Hydrodynamic size of Al_2O_3 NP dispersion in filtered lake water at the 0th h using a dynamic light scattering particle size analyzer. (D) Hydrodynamic size of Al_2O_3 NP dispersion in filtered lake water at the 4th h using a dynamic light scattering particle size analyzer. (E) Size distribution of nanosized natural colloidal particles in filtered lake water using a dynamic light scattering particle size analyzer.

when dispersed in Millipore water (Figure 1B). Three different size ranges (i.e., 62−65 nm, 78−88 nm, and 115−140 nm) were obtained through the hydrodynamic size distribution analysis of particles at the zeroth and fourth hour in sterile lake water [*z*-average size of 83 ± 1.3 nm at the zeroth hour and 85 ± 1.2 nm at the fourth hour] (Figure 1C,D). The additional size range at 115−140 nm could be related to the presence of nanosized suspended particles in lake water. The hydrodynamic size analysis of filtered lake water (before nanoparticle addition) confirmed the presence of particles at 115−139 nm range (Figure 1E).

It can be inferred from the size distribution analysis that Al_2O_3 NP were stable (against aggregation) in the lake water matrix for a period of 4 h. The lake water matrix played a significant role in stabilizing the Al_2O_3 NP as reported earlier for other metal oxide

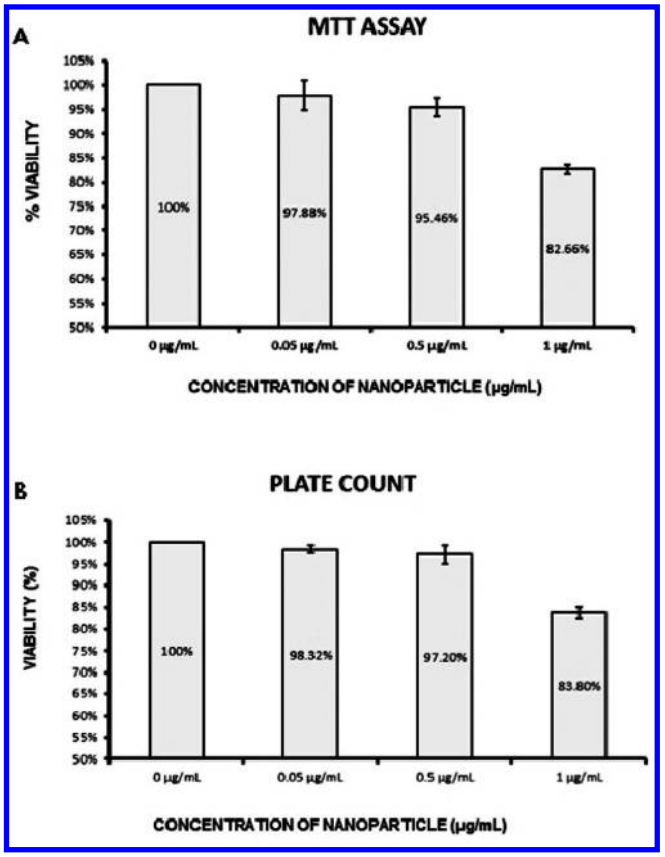

Figure 2. (A) Graph showing % viability of bacterial population on treatment with increasing concentrations of Al_2O_3 NP measured by the MTT assay. (B) Graph showing % viability of bacterial population on treatment with increasing concentrations of Al_2O_3 NP measured by plate counting. # Statistically significant based on one-way ANOVA followed by Dunnett's post-hoc test at $p < 0.05$.

nanoparticles (TiO_2, ZnO, and CeO_2).[5] During short-term exposure, presumably Al_2O_3 NP were stabilized by the nanosized suspended organic matter in the filtered lake water and exerted a nano-size effect on the exposed biota.

Cell Viability Evaluation. The dominant bacterial species in the lake water was isolated and identified to be *Bacillus licheniformis* by 16S rRNA analysis (length of the sequence, 1462 bp; homology, 99%). The cell viability was evaluated by measuring the number of viable cells present in the test sample after 2 h of nanoparticle interaction. Decrease in the viability of *B. licheniformis* was found to be 17.3% upon exposure to 1 μg/mL, whereas 0.5 μg/mL and 0.05 μg/mL showed a 4.5% and 2.1% decrease in viability, respectively, as compared to the control (MTT assay, Figure 2). The plate count also corroborated with the MTT assay showing a 16.2% decrease in viability at 1 μg/mL, whereas 0.5 μg/mL and 0.05 μg/mL resulted in a 2.8% and 1.7% decrease in viability, respectively, as compared to the control (Figure 2). One-way ANOVA analysis followed by Dunnett's post-hoc test confirmed that the effect of Al_2O_3 NP on cell viability (by the MTT assay and plate count) was significantly different ($p < 0.05$) for 0.5 μg/mL and 1 μg/mL as compared to the control.

These studies imply that a dose dependent toxic nature of Al_2O_3 NP could be valid even at low exposure levels. To the best of our knowledge, the minimum reported Al_2O_3 NP concentration showing a cytotoxic effect in the prokaryotic system is 20 μg/mL[8]

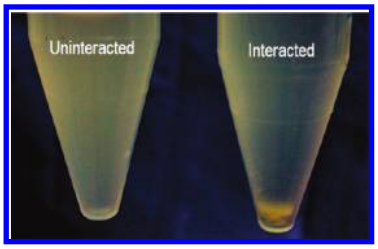

Figure 3. Flocculation of bacterial cells on interaction with Al_2O_3 NP for 2 h in the test; the uninteracted cells do not show any flocculation after the same time period.

and that for the eukaryotic system is 0.5 μg/mL in Chinese hamster ovary (CHO-K1) cells.[23]

There can be two major underlying mechanisms for the observed cytotoxicity, namely, (i) inherent chemical toxicity of Al_2O_3 NP based on the chemical composition, e.g., release of (toxic) Al^{3+} ions, or (ii) due to stress or stimuli caused by the surface, size, and/or shape of the particles.[24]

Surface Charge, Al_2O_3NP Attachment, and Flocculation. Surface attachment of nanoparticles can play a decisive role in bacterial toxicity. The average surface area of a representative single bacterium was calculated to be 2.8 μm^2 (Supporting Information, Figure S1), which corresponds to the surface area of the bacterial suspension, i.e. 4.1 m^2/L.[25] This indicates a high adsorption capacity of bacteria and prospects of Al_2O_3 NP attachment to the cell surface. The zeta potential of nanoparticle dispersion in lake water at pH 7.0 was found to be +10.9 ± 2.51 mV, and that of the bacterial cell was −16.4 ± 3.28 mV. The negatively charged bacterial surfaces would electrosterically attract the positively charged Al_2O_3 NP and Al_2O_3 NP being much smaller in size than the bacterial cells would envelope the bacteria cell, neutralizing the bacterial surface charge, and, as a result, forms large aggregates. We have observed flocculation of the bacterial cell suspension on interaction with 1 μg/mL Al_2O_3 NP, which was not observed in the untreated cells (Figure 3). A similar charge based aggregation and flocculation mechanism was reported earlier for oxide nanoparticles when interacted with bacteria.[8]

Cell Wall Damage by Al_2O_3NP. The flocculation and aggregation data suggested that the bacterial toxicity could be due to the surface attachment of Al_2O_3 NP on to the cells. The attachment theory was also supported by comparison of the FT-IR spectral data (details in Figure S2, Supporting Information) of treated cells with those of the untreated ones. A conspicuous vibrational frequency band at 639 cm^{-1} in the treated sample confirmed the presence of AlO_6 condensed octahedral units, forming γ-Al_2O_3 structure.[26−28]

The SEM image showed noticeable damage on the cell surface in the treated bacteria (Figure 4A,B). This was further confirmed by the lipid peroxidation assay (Supporting Information).

Al^{3+} Ion Mediated Chemical Toxicity. Different prevalent inorganic and heavy metal ions in the lake water matrix were quantified (Table S1, Supporting Information). In the control experiment (sterile lake water devoid of the particles), the basal concentration of the Al^{3+} ion (0.289 ± 0.03 μg/mL) was found to be nontoxic to the bacteria.

The filtered Al_2O_3 NP suspension, devoid of any particles and containing only dissolved ions (obtained after 2 h of incubation; confirmed through hydrodynamic size analysis), failed to induce any toxic effect to the bacteria upon exposure. The Al^{3+} ion concentration in Al_2O_3 NP free suspension was 0.292 ± 0.06 μg/mL,

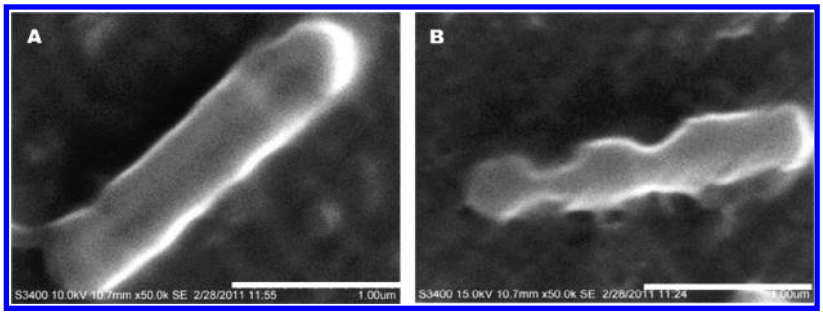

Figure 4. (A) Scanning electron micrograph of an untreated bacterial cell showing no cell damage. (B) Scanning electron micrograph of 1 μg/mL Al_2O_3 NP treated bacterial cell showing extensive damage.

not significantly different [Student's t test at $p < 0.05$] compared to the basal concentration in lake water. Therefore, an ion mediated toxicity by the released Al^{3+} ion from Al_2O_3 NP during the course of the experiment (2 h) can be ruled out. Reports on the insoluble nature of Al_2O_3 NP also corroborates with this finding.[8]

CONCLUSIONS

The Al_2O_3 NP showed a significant toxicity toward a dominant microbial species (*Bacillus licheniformis*) at a low concentration (1 μg/mL). The stability of Al_2O_3 NP in filtered lake water, presumably, owing to the presence of nanosized natural colloids, was observed to influence its short-term toxicity. The decrease in cell viability was not due to the release of Al^{3+} ions in the dispersion. The surface charge based interaction between the bacteria and NP resulted in aggregation and subsequent flocculation of the bacterial cells, thus causing toxicity. The cell wall damage confirmed through SEM and the lipid peroxidation assay could also have contributed to it. Comprehending the mechanistic aspects of this phenomenon entails further detailed research.

ASSOCIATED CONTENT

Supporting Information. The preparation of Al_2O_3 NP dispersion in lake water; table of different inorganic and heavy metal ions present in lake water; toxicity studies including details of dominant bacteria isolation, characterization, and identification; experimental design for bacteria-Al_2O_3 NP interaction; methods for standard plate count and lipid peroxidation assay; method for zeta potential measurement; sample preparation for SEM; method for FT-IR analysis; FT-IR spectra for Al_2O_3 NP treated and untreated bacterial cells; and figure of bacterial cells by SEM. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

NP, nanoparticle; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; DMSO, dimethyl sulphoxide; CFU, colony forming unit; EC_{50}, median effective concentration; LC_{50}, median lethal concentration

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