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Diluent Mitigates the Inhibitory Effect of Zinc Oxide Nanoparticles on *Escherichia coli* and *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration between both authors. Authors SE and NA designed the study, performed the statistical analysis and wrote the protocol. Author SE wrote the first draft of the manuscript. Both authors managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

The effects of zinc oxide nanoparticles on *Escherichia coli* and *Staphylococcus aureus* in deionized water, normal saline and phosphate buffered solution were investigated based on culture-dependent growth response. After 6 h incubation in phosphate buffered solution, the 10, 100 and 1000 µg mL^{-1} treatment resulted in a range of 1.0 to 1.5 log reduction in growth of *E. coli* and *S. aureus* compared with the control. In the normal saline, 1.1 to 3.3 and 1.1 to 3.6 log reduction whereas 1.2 to 4.1 and 1.2 to 3.8 log reduction in deionized water was observed for *E. coli* and *S. aureus* respectively. Inhibitory effect of ZnO nanoparticles on the organisms in the diluents was concentration-dependent with 1.8 to 2.6 times higher viability of *E. coli* and *S. aureus* in PBS compared to the normal saline and deionized water, but the difference was significant (P = .05). The evidence suggests differential susceptibility of *E. coli* and *S. aureus* to ZnO nanoparticles in diluents routinely used in microbiological assay and raises concern on the challenges of interpreting the inhibitory effect in culture-dependent microbiological analysis. Compared with

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deionized water and normal saline, phosphate buffered solution mitigated the inhibitory effect of ZnO nanoparticles on the growth dynamics and population density of *E. coli* and *S. aureus.*

Keywords: ZnO nanoparticles; Escherichia coli; Staphylococcus aureus; diluent; inhibitory effect.

1. INTRODUCTION

Engineered nanoparticles (ENPs) are materials with nanometer scale dependent properties and function, synthesized and used in diverse industrial processes and consumer product manufacturing [1,2]. Among the ENPs, metal oxide nanoparticles are generally preferred as a result of their unique physical, chemical and biological properties that are different from their bulk counterpart [3,4]. These properties enable ENPs to be widely applied in consumer products especially in the health and fitness sector [2]. Of particular interest is zinc oxide (ZnO) nanoparticles used as skin protectants in sunscreen [5] and antimicrobials [6]. ZnO nanoparticles has broad spectrum bactericidal properties [6,7] but the mechanism of action is yet to be fully elucidated. However, two key mechanisms proposed for the ENPs toxic effect on microorganisms are the oxidation stress from reactive oxygen species (ROS) causing lipid peroxidation [8] and ions interacting with key biotic receptors such as cell membrane/wall, protein and DNA [6,9]. Other factors such as size of ENPs, presence of divalent cations/anions and surface charges [10], bacterial cell wall composition/charge [11], and capping agent which repels ENPs by electrostatic, steric or electrosteric forces enables them to resist forming aggregates [12] can either enhance or attenuate ENPs bactericidal effect.

Although laboratory controlled selective toxicity of size- and dose-dependent pristine ZnO nanoparticles has been demonstrated [6,7], it is unclear if the same size/concentration or conditions are obtained after pre-treatment of ZnO nanoparticles to exert similar acute effect. Thus, the negative impact of nanoparticles on microbial system is usually evaluated whereas the *in vitro* effect of diluent on the pristine nanoparticles can be overlooked. However, there is no consensus on the interaction between bacterial cell wall composition and its influence on ENPs toxic effect. Indeed, the notion that bacterial cell wall has no influence on ENPs effect [13] contrasts with the view that it plays a significant role in reducing or enhancing ENPs toxic effect [11].

It has been perceived that analytical procedures and pre-treatment of samples such as sonication [14] and drying for electron microscopy offer significant challenges as a result of the alteration in ENPs physicochemistry and plausibly the experimental outcome [15]. If diluents used in microbiological assay interferes with the outcome of microbiological assay, then the implications are profound and potentially disturbing in the determination of inhibitory dose of the toxicant. Thus, inhibitory effect can either be enhanced or minimized as a result of diluent-ENPs interactions. In the light of the concerns and gaps in knowledge, our goal was to assess ZnO nanoparticles inhibitory effect in different diluents on model Gram positive and negative microorganism. Specifically, we tested whether diluents such as deionised water, normal saline and phosphate buffered solution used in routine microbiological assay can influence ZnO nanoparticles inhibitory properties on the growth response of *Escherichia coli* and *Staphylococcus aureus and* the significance of such interactions.

2. MATERIALS AND METHODS

2.1 Characteristics of Zinc Oxide Nanoparticles

Zinc oxide (ZnO) nanoparticles were purchased from *NanoA*mor (Nanostructured and Amorphous Materials Inc. Texas, USA) and the properties are as characterised by the manufacturer. The properties include an average particle size of 20 nm, specific surface area of 50 m^2/g having a bulk density of 0.3–0.45 g/cm^3 and a true density of 5.606 g/cm^3. The material is an odourless powder, 99.5% pure having a nearly spherical morphology, insoluble in aqueous medium with a melting point of 1975℃. Toxicological significance of the particles are not fully known although there are evidence of mutagenic and inhibitory effects on biological systems.

2.2 Identification of Test Organism and Viability Assay

We obtained the test organism from the University of Uyo Teaching hospital and its identity was confirmed by subculturing at

35±0.2℃ on chromogenic *E. coli* /coliform selective medium (Oxoid) and also on modified Lauryl tryptose broth with MUG and added tryptophan. *E. coli* was differentiated from other coliforms (pink colonies) by their typical purple colonies and blue-green fluorescence on the respective growth medium. *Staphylococcus aureus* was confirmed on mannitol salt agar. The cells were harvested, subcultured and optical density (OD) adjusted to 1.0 at 600 nm after incubation. The culture aliquots were spun at 5,000 g for 5 min and the supernatant discarded. The static toxicity test in which organisms were placed into one of a series of exposure concentrations of ZnO nanoparticles in diluents routinely used in microbiological assay was used. The cells were resuspended in deionized water, physiological saline (0.85% of NaCl) and phosphate buffered solution containing 8 g L^{-1} NaCl, 2 g L^{-1} KCl. 1.44 g L^{-1}, Na_2PO_4, 0.24 g L^{-1}. The assay was done in triplicate and spiked with 10, 100 and 1000 µg mL^{-1} concentrations of ZnO nanoparticles. The ZnO nanoparticles in each diluent were magnetically stirred at 150 rpm for 1 h without addition of dispersant. Tubes containing the diluents without ZnO nanoparticles were taken as the control and all were incubated at 35±0.2℃ for 6 hours.

2.3 Determination of Bacterial Growth Kinetics

We measured bacterial growth in the presence of ZnO nanoparticles photometrically from the optical density of the broth as previously described [16,17]. 1.0 ml of sample from each treatment was vortexed for 30 seconds, sonicated for 1 min and allowed to stand for 2 min. The samples were inoculated onto 100 ml of Nutrient broth No. 2 (Oxoid) and incubated at 35±0.2℃ and constantly agitated at 170 rpm. At intervals of 1 hour, 3 ml of broth culture withdrawn from each flask and optical density (OD) measured at 600 nm for 6 h were used to determine effect on bacterial growth.

2.4 Determination of Microbial Population Density

To assess the effect of ZnO nanoparticles on the viability of *E. coli* and *S. aureus,* 1.0 ml aliquot of the bacterial suspension was serially diluted and 1.0 ml of 10^{-5} dilution in each series was pour-plated in triplicate onto Mueller-Hinton, Plate count agar mannitol salt agar and chromogenic *E. coli* /coliform selective medium (Oxoid). Colony-forming units (CFUs) from a set of triplicate sample dilutions incubated at 35±0.2℃ for 24 h were enumerated and the results are expressed as CFU mL^{-1}. The percentage survival of culturable bacterial density was determined using the log-transformed CFU mL^{-1} values [18] and the formula:

$$\text{Percentage survival (\%S)} = (CFU_{exposed} / CFU_{control}) \times 100$$

Where $CFU_{exposed}$ is the colony forming units from the ZnO spiked samples and $CFU_{control}$ is the total colony forming units from the unspiked diluent (control).

2.5 Statistical Analysis

Analysis of variance (ANOVA) and Kruskal-Wallis test was performed on log-transformed data using *Statistica* software® version 12 (Statsoft, Tulsa, OK, USA). Results are presented as mean ± standard deviation with levels of significance maintained at 95% for each test.

3. RESULTS AND DISCUSSION

3.1 ZnO Nanoparticles Effect on Bacterial Growth Dynamics

The antibacterial activity of ZnO nanoparticles in different diluents on *E. coli* and *S. aureus* is presented in Fig. 1. The result suggests diluent- and concentration-dependent inhibitory effect on *E. coli* and *S. aureus* after 6 h of incubation. A difference of 1.3 to 1.6 and 1.3 to 1.9 times reduction in growth was recorded for *E. coli* and *S. aureus* respectively across the different ENPs concentrations in PBS compared to the control. In the normal saline, the difference in growth reduction was 1.3 to 14 and 2.2 to 9.3 times lower, whereas in deionized water, a range of 1.6 to 14 and 1.3 to 7.4 times reduced growth of *E. coli* and *S. aureus* respectively was obtained. Reduced growth were based on the microbial density in diluent spiked with ZnO nanoparticles relative to the unspiked control of each diluent.

At 10 µg mL^{-1} of ZnO nanoparticle, the difference between *E. coli* and S. aureus growth reduction was low but significant (P = .05) in the three diluents. ZnO nanoparticles formed aggregates in deionized water compared to the homogenous colloidal suspension in normal

saline and PBS, but inhibitory effect on the organisms in deionized water was more pronounced than in the normal saline and PBS (Fig. 1). In a study by Reinsch et al. [19], aggregated silver oxide nanoparticles were more toxic to *E. coli* than the dispersed form due to the release of ions. Similarly, the result of ZnO nanoparticles aggregates in deionized water exhibited inhibitory effect on the test organisms and indicates that the release of ion was a prominent mechanism to exert the toxic effect. ZnO nanoparticles are positively charged between pH 6 to 8, negatively charged between pH 10 to 12 or undergo partial dissolution at acidic pH to form Zn^{2+} [11,20]. At the homeostatic pH and ionic composition of the diluents, the ion produced by ZnO nanoparticles and the differential surface charges of the organisms exacerbated the negative effect on the *E. coli and S. aureus.* Overall, the inhibitory effect of ZnO nanoparticles was mitigated in the presence of PBS, but the causal relationship between the PBS components and the ZnO nanoparticles that contributed to the effect is unclear at the moment.

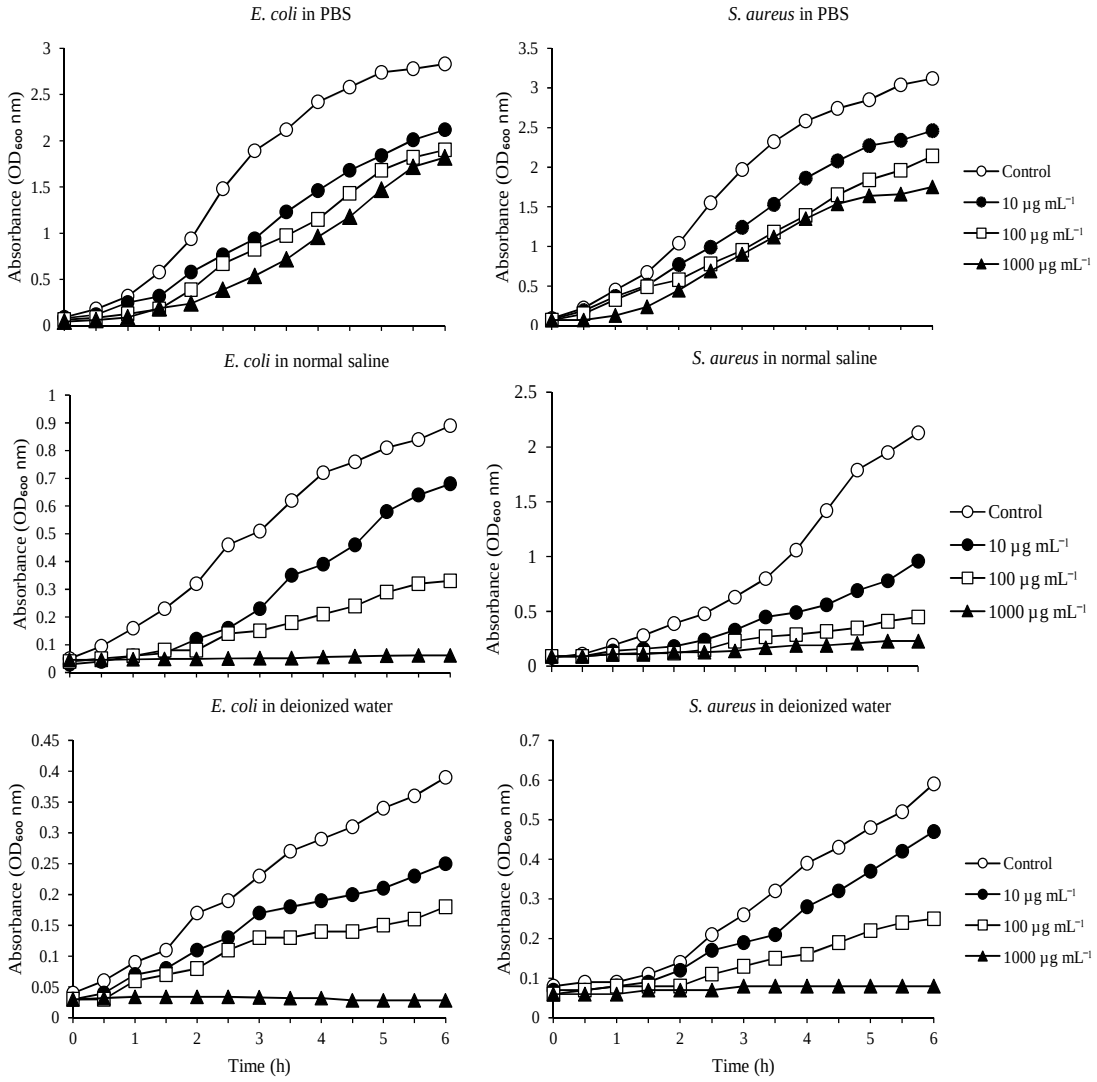

Fig. 1. Effect of ZnO nanoparticles on the growth response of *E. coli and S. aureus* in deionised water, normal saline and phosphate buffered solution

3.2 Effect of ZnO Nanoparticles on Viability and Survival of *E. coli* and *S. aureus*

Culture-dependent result of *E. coli* and *S. aureus* viability in deionised water, normal saline and phosphate buffered solution spiked with ZnO nanoparticles after 6 hours incubation is represented in Fig. 2. The inhibitory effect of ZnO nanoparticles in the diluents was concentration-dependent and followed the pattern PBS < normal saline < deionized water. In PBS, the 10 to 1000 µg mL^{-1} treatment resulted in 1.0 to 1.5 log reduction for *E. coli* and *S. aureus* compared with the control. In normal saline, a range of 1.1 to 3.3 and 1.1 to 3.6 log reduction, whereas 1.2 to 4.1 and 1.2 to 3.8 log reduction in deionized water was observed for *E. coli* and *S. aureus* respectively. Generally, ZnO nanoparticles adverse effect is closely related to the release of zinc ions (Zn^{2+}) and the bioavailable dose [21] responsible for lipid peroxidation through the induction of oxidative stress, cell membrane damage and cytoplasmic leakage [22].

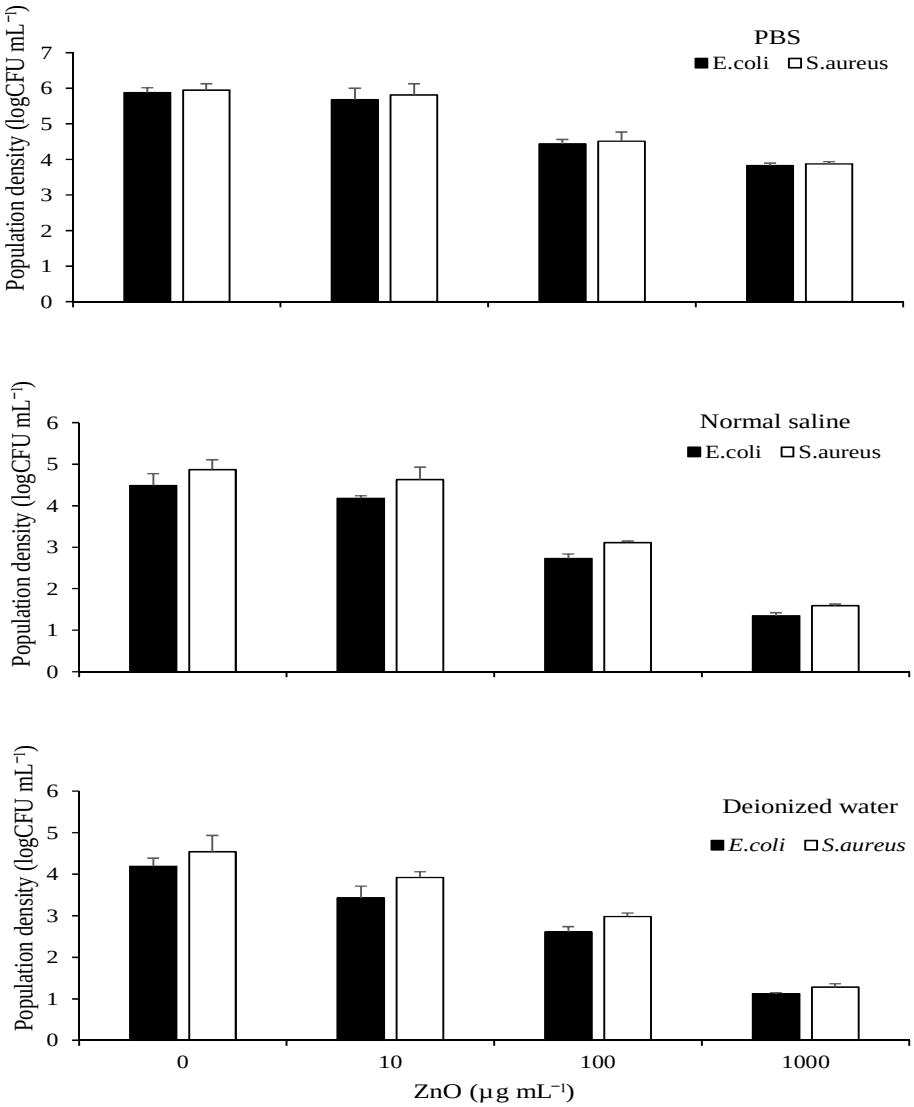

Fig. 2. Effects of ZnO nanoparticles on the population densities of *E. coli* and *S. aureus* in routinely used microbiological diluents

Also, the oxidative stress-induced cell damage is bacterial species-dependent [7]. In this study, the ZnO nanoparticles concentration-dependent, stress-induced growth inhibition was more pronounced on *E. coli* than *S. aureus* in the normal saline and deionised water and suggests the influence of the bacterial cell wall interaction with Zn^{2+}. Furthermore, the results indicate that at 1000 µg mL^{-1} ZnO nanoparticles concentration, a difference of 1.8 to 2.6 times higher viability of *E. coli* and *S. aureus* in PBS was recorded compared to the normal saline and deionized water, but the difference was significant (*P* = .05).

The effect of ZnO nanoparticles on the percentage survival of *E. coli* and *S. aureus* is shown in Fig. 3. The results indicate a 1.5 times difference in percentage reduction between 10 and 1000 µg mL^{-1} on the survival of *E. coli* and *S. aureus* in PBS compared with the control. Survival of *E. coli* and *S. aureus* in normal saline differed by 3.1 and 2.9 times, whereas a difference of 2.9 and 3.1 respectively was obtained in deionised water, but the difference was statistically significant (*P* = .05). Furthermore, the result suggests concentration-dependent and graded inhibitory effect influenced by the composition of the different diluents.

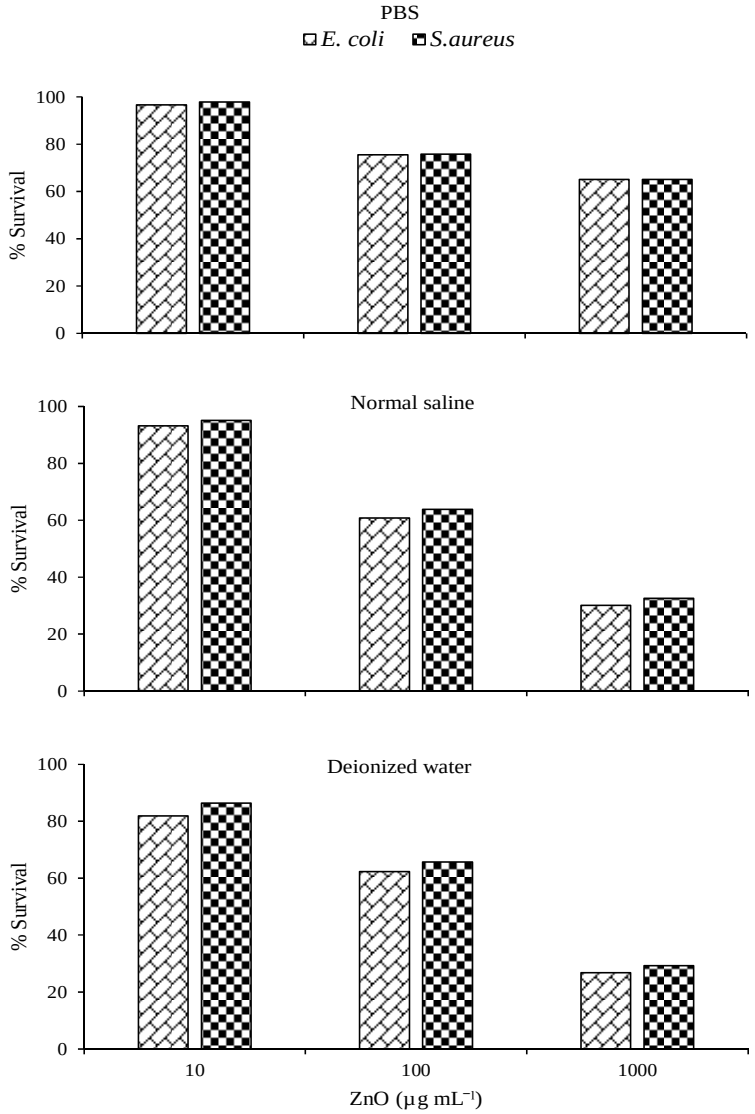

Fig. 3. Percentage survival of *E. coli* and *S. aureus* exposed to ZnO nanoparticles in deionised water, normal saline and phosphate buffered solution

The effect of ZnO nanoparticles on the growth and survival of *E. coli* and *S. aureus* in the different diluents varied across the concentrations tested. Of all the diluents assessed, PBS was by far the best mitigator of ZnO nanoparticles inhibitory effect with the lowest survival of 65% for both organisms at 1000 µg mL^{-l}. Indeed, PBS components reduced the interaction of ZnO nanoparticles with the organisms during incubation to elicit the inhibitory effect, hence survival of the organisms was more pronounced in PBS than in normal saline and deionised water. However, it was difficult to determine effective dose of the ZnO nanoparticles in contact with the organisms in the different diluent, in part as a result of interaction with the component ions in the diluents, and in part to the inadequacy of the analytical procedures.

4. CONCLUSION

In conclusion, our data suggest that the composition of diluent used in routine microbiological assay such as PBS attenuated the inhibitory effect of ZnO nanoparticles on the growth of *E. coli* and *S. aureus.* The model organisms suggest that the cell wall/membrane properties are involved in the interaction which enhanced or attenuated the inhibitory effect of ZnO nanoparticles on bacterial growth. The result raises concern on how to assess and interpret the inhibitory effect of ZnO nanoparticles on gram positive and negative bacterial species or extrapolate the result from one diluent or organism to another without bias. Overall, phosphate buffered solution mitigated the inhibitory effect of ZnO on the growth of *E. coli* and *S. aureus* in relation to normal saline and deionised water.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Pan X, Redding JE, Wiley PA, Wen L, McConnell JS, Zhang B. Mutagenicity evaluation of metal oxide nanoparticles by bacterial reverse mutation assay. Chemosphere. 2010;79:113-116.
2. Woodrow Wilson Database. Project on Emerging Nanotechnologies: Consumer products inventory. Woodrow Wilson International Center for Scholars Washington DC; 2011.
   Available:http://www.nanoproduct.org/inventories/consumer/
   (Accessed June 28, 2015)
3. Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, Philbert MA, Ryan J, Seaton A, Stone V, Tinkle SS, Tran L, Walker NJ, Warheit DB. Safe handling of nanotechnology. Nature. 2006;444(7117):267-269.
4. Eduok S, Hendry C, Ferguson R, Martin B, Villa R, Jefferson B, Coulon F. Insights into the effect of mixed engineered nanoparticles on activated sludge performance. FEMS Microbiology Ecology. 2015;91.
   DOI: 10.1093/femsec/fiv082
5. Schrand AM, Rahman MF, Hussain SM, Schlager JJ, Smith DA, Syed, AF. Metal-based nanoparticles and their toxicity assessment. National Center for Toxicological Research, Jefferson, USA; 2010.
6. Xie Y, He Y, Irwin PL, Jin T, Shi X. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against *Campylobacter jejuni.* Appl Environ Microbiol. 2011;77(7):2325-31.
7. Wu B, Wang Y, Lee YH, Horst A, Wang Z, Chen D, Sureskumar R, Tang YJ. Comparative eco-toxicities of nano-ZnO particles under aquatic and aerosol exposure modes. Environ Sci. Technol. 2010;44:1484-1489
8. Choi O, Hu Z. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. Environ Sci Technol 2008;42:4583-4588.
9. Bottero JY, Auffan M, Rose J, Mouneyrac C, Botta C, Labille J, Masion A, Thill A, Chaneac C. Manufactured metal and metal-oxide nanoparticles: Properties and perturbing mechanisms of their biological activity in ecosystems. C. R. Geosci. 2011; 343(2−3):168−17.
10. Li X, Lenhart JJ, Walker HW. Dissolution-accompanied aggregation kinetics of silver nanoparticles. Langmuir. 2010;26(22): 16690−16698.
11. Jin T, Sun D, Su JY, Zhang H, Sue H-J. Antimicrobial efficiency of zinc oxide, quantum dots against *Listeria monocytogenes*, *Salmonella enteritidis*, and *Escherichia coli* 0157:H7. J Food Sci. 2009;74:46-52.

12. Hotze EM, Phenrat T, Lowry GV. Nanoparticle aggregation: Challenges to understanding transport and reactivity in the environment. J Environ Qual. 2010;39(6):1909−1924.
13. Chudasama B, Vala AK, Andhariya N, Mehta RV, Upadhyay RV. Highly bacterial resistant silver: Synthesis and antibacterial activities. J Nanopart Res. 2010;12:1677-1685.
14. Smith CJ, Nedwell DB, Dong LF, Osborn AM. Diversity and abundance of nitrate reductase genes (narg and napa), nitrite reductase genes (nirS and nrfA), and their transcript in estuarine sediments. Appl Environ Microbiol. 2007;73:3612-3622.
15. Tiede K, Hassellov M, Breitbarth E, Chaudhry Q, Boxall ABA. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessment for nanoparticles. J Chromat A. 2009;1216:503-509.
16. Pal S, Tak YK, Song JM. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticles? A study of the gram-negative bacterium *Escherichia coli.* Appl Environ Microbiol. 2007;73(6):1712-1720.
17. Das MR, Sarma RK, Saikia R, Kale VS, Shelke MV, Sengupta P. Synthesis of silver nanoparticles in an aqueous suspension of graphene oxide sheets and its antimicrobial activity. Colloids and Surfaces B: Biointerfaces. 2011;83(1):16-22.
18. Diao M, Yao M. Use of zero-valent iron nanoparticles in inactivating microbes. Wat Res. 2009;43:5243-5251.
19. Reinsch BC, Levard C, Li Z, Ma R, Wise A, Gregory KB, Brown GE Jr, Lowry GV. Sulfidation decreases silver nanoparticle growth inhibition effects for *Escherichia coli*. Environ Sci Technol. 2012;46: 6992−7000.
20. Brayner R, Dahoumane SA, Yéprémian C, Djediat C, Meyer M, Couté A, Fiévet F. ZnO nanoparticles: Synthesis, characterization, and ecotoxicological studies. Langmuir. 2010;26(9):6522-6528.
21. Crane M, Handy RD, Garrod J, Owen R. Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. Ecotoxicol. 2008;17(5):421-437.
22. Xia T, Kovochich M, Liong M, Madler L, Gilbert B, Shi H, Yeh JI, Zink JI, Nel AE. Comparision of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano. 2008;2:2121-2134.

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