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Importance of sample pretreatment on the bacterial bioassay for toxicity of ZnO nanoparticles

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Abstract: (Eco)toxicological assessment of engineered nanoparticles has largely restricted to laboratory conditions, and contribution to the environmental matrix mostly ignored. Thus, the lack of reflection of the environmental matrix makes it harder to evaluate the ecotoxicity of nanoparticles and its mechanism. Therefore, sample pretreatment has a vital role for appropriate (eco)toxicological assessment and reflection of the environmental matrix. The present study aims to investigate the effect of different sample pretreatment strategies on the (eco)toxicity of ZnO nanoparticles and its mechanism. For this purpose, three sample pretreatments and related bacterial bioassay procedure was examined. Moreover, the impact of the sample pretreatment on the toxicity mechanism was investigated using the physicochemical parameters of ZnO nanoparticles and antioxidant responses of bacteria. The results showed that the sample pretreatment affected the physicochemical properties of ZnO nanoparticles, inhibition level and inhibition pathway.

Keywords: Sample preparation; nanoparticles; bacterial bioassay; matrix effect; seawater.

1. Introduction

Although sample pretreatment is often the bottleneck in an analysis, it is important in all aspects of biological, chemical, materials, and surface analysis. It aims to bring the samples in a suitable form, ready for analysis, and to minimize the sample degradation during analysis. Also, in order to apply an analytical procedure, it is important to avoid interferences between the analyte and the components of the matrix. Once the sample pretreatment is complete, the analysis is carried out by different types of analysis, depending on the information to be acquired; toxicological, physicochemical, etc. [1-4]. Despite having importance on quality and fate of analysis, it did not

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receive much attention in the environmental fate of nanoparticles (NPs) and there is still much to be done in terms of methodological aspect [5].

The growing production and wide application of NPs have increased their release in the environmental systems (e.g. air, water, soil), and the main sink of the NPs in the environment can be seen in water systems, such as seawater, sewage water. With the increasing release of NPs, there are various attempts to evaluate their fate in the environment [6-8]. Currently, understanding the behavior of NPs in the environmental systems and interaction of NPs with environmental components is still unknown due to no established methodology. Only limited number of studies evaluated the matrix effect on the particle size and zeta potential of NPs and their relation to bacteria [6,9-11]. In most of these studies, the matrix was mostly artificial (e.g. addition of NaCl, Na_2SO_4 in distilled-deionized water), or only distilled-deionized/ultra-pure water. In addition to this, a few studies used real environmental matrix (e.g. seawater, wastewater, extracted air particulate matter) [2,9-13]. The studies proved that both artificial and real environmental matrix influenced the surface chemistry, particle size and zeta potentials of NPs.

Till to date, to investigate the behavior of NPs in environmental systems, bacterial bioassay (i.e. *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*) are good test model, because they exist in large numbers and have important roles in the environment. The interaction between bacteria and NPs may provide information about the impact of NPs in the environment. For instance, bacteria test model allows us to study the toxicity of NPs against the cell/organism, and to examine them in different environmental matrices [9-13, 17]. According to the studies conducted on bacteria, the presence of NPs in the environment can positively or negatively affect the functions of the microorganisms [6, 9-13, 18-27]. Despite the large number of studies (>40000) dealing with the ecotoxicity of NPs, it is still not possible to establish the interaction between NPs and the environment. This is mainly due to the fact that the available studies have important shortcoming about the experimental design, such as lack of physicochemical characterization of NPs under environmentally realistic conditions and/or the disconnection between the environmental or experimental conditions for both toxicity assay and physicochemical characterization [14, 28-34]. All these issues also induce many analytical questions. To diminish these shortcomings, new studies conducting toxicity procedures under environmental conditions and applying appropriate sample pretreatment methods for the physicochemical characterization are crucial for linking the environmental systems with toxicity procedures.

The different matrixes can change the NPs surfaces because the surface of NPs may include organic capping agents or may purify with the surrounding matrix [2,9-13, 35,36]. Unfortunately, examination of the extensive surface chemistry has been mostly ignored under various experimental conditions. The surface chemistry can influence their biological effects and also the particle size, zeta potential, agglomeration.

Thus, in order to make realistic assumptions for the fate of ZnO NPs on the environmental systems, the effect of the sample pretreatment strategies of ZnO NPs on the toxicity and its mechanism was evaluated with and without seawater conditions.

2. Experimental

2.1. Materials and Instrumentation

ZnO NPs were obtained from Nanografi (Ankara, Turkey). All chemicals used in the experiments were of analytical grade and obtained from Merck (Darmstadt, Germany). Biochrom Libra S70 (Cambridge, UK) spectrophotometer was equipped for all measurements in optical density and biochemical assays.

Escherichia coli (ATCC 35218, *E. coli*), *Pseudomonas aeruginosa* (ATCC 27853, *P. aeruginosa*) and *Staphylococcus aureus* (ATCC 25923, *S. aureus*) served as a model organism and taken from The American Type Culture Collection (ATCC). Frozen strains were activated according to producer data sheet, which requires keeping them at 37 °C for 3 h in Tryptic soy broth (Merck) and culturing on Nutrient agar (Merck) prior to the toxicity assessment.

2.2. Procedures of Sample Pretreatment of ZnO Nanoparticles

Applied sample pretreatment methods and related toxicity procedures were summarized in Table 1 [2]. For this purpose, firstly, 2.0 mg of ZnO NPs were weighed and combined with 20 mL treatment (matrix) solution in 50 mL flask. Seawater was selected as an environmental matrix. Distilled-deionized water (Milli-Q, Millipore, USA) and seawater were used as treatment solutions. Chemical properties of seawater characterized according to the standard methods given in Supplementary Table S2, and results were shown in Supplementary Table S3. In order to avoid the formation of precipitation/agglomeration, the mixtures containing ZnO NPs were stirred at 250 rpm for 24 h. Subsequently, the mixture was centrifuged to separate NPs from the solution part and dried in an oven at 90ºC to dry.

Table 1. Applied sample pretreatment methods and related toxicity procedures

Sample pretreatment of NPs		
Method-I	*Method-II*	*Method-III*
No treatment: • Dried at 90ºC	Treatment with distilled water: • 10% (w/v) NPs prepared in distilled water • Shaken 24 h at 250 rpm • Removed the aqueous part • Dried at 90ºC	Treatment with seawater • 10% (w/v) NPs prepared in seawater • Shaken 24 h at 250 rpm • Removed the aqueous part • Dried at 90ºC

Procedure of bacterial bioassay for NPs toxicity			
Procedure 1	*Procedure 2*	*Procedure 3*	*Procedure 4*
Sample Preparation: Method I Procedure: • Broth for inoculation of bacteria prepared with ***distilled water*** • 24 h incubation • Optical density measurement at 600 nm	Sample Preparation: Method I Procedure: • Broth for inoculation of bacteria prepared with ***seawater*** • 24 h incubation • Optical density measurement at 600 nm	Sample Preparation: Method II Procedure: • Broth for inoculation of bacteria prepared with ***distilled water*** • 24 h incubation • Optical density measurement at 600 nm	Sample Preparation: Method II Procedure: • Broth for inoculation of bacteria prepared with ***distilled water*** • 24 h incubation • Optical density measurement at 600 nm

2.3. Toxicity Assessment

To examine the toxicological behavior of the ZnO NPs, bacteria model system was used and procedures were explained in Table 1. Each procedure without ZnO NPs was applied as blank or control of the procedure. All samples were prepared in five replicates. Averages of each of the five samples or plates are reported in the results, figures, and tables. Error bars in the results represent the standard deviation of the five measurements. Finally, all samples were incubated in a dark oven at 37ºC for 24 h. After the incubation time, the viability was measured by the optical density measurement at 600 nm [2].

2.4. Physicochemical Properties of ZnO Nanoparticles

After the sample pretreatments, the treated ZnO NPs were characterized by surface chemistry, morphology, zeta potential, and particle size.

Surface chemistry was investigated using Fourier-transform infrared (FTIR) spectrometry (VERTEX 70 ATR, Bruker, Germany). The FTIR analysis was acquired in the range of 4000 to 500 cm^{-1} to investigate the effect of sample pretreatment on the surface chemistry of ZnO NPs.

Zeta potential and particle size were measured using Dynamic light scattering (DLS, Malvern Zetasizer Nano ZS, Worcestershire, UK). After 5 minutes of sonication, particle size measurements were carried out in a disposable sizing cuvette and zeta potential analysis was conducted by injecting 1 mL of sample in a folded capillary cell.

The morphology and chemical composition of ZnO NPs was determined using a Scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDX, QUANTA FEG 250, FEI, Thermo Fisher Scientific, Oregon, USA).

2.5. Biochemical Responses of ZnO Nanoparticles

To investigate the antioxidant capacity and antioxidant enzymes (Dehydrogenase, Superoxide dismutase, Catalase) related to oxidative stress, formed bacteria colonies on nutrient broth were transferred and suspended into saline water (0.9% NaCl) until they reached to an optical density corresponding to $1x10^8$ CFU/mL. Then prepared samples were immediately used for the analysis of the antioxidant capacity and enzymes.

Antioxidant capacities were determined by CUPRAC method [35]. Briefly, sample solutions were mixed with 0.01M $CuCl_2$, $7.5x10^{-3}$ M Neocupreine, and 1 M CH_3COONH_4. Sample solutions were measured after the 30 min waiting period and Trolox was used as a standard.

Dehydrogenase (DeH) activities were determined according to the method described in the literature [38]. The method basically involves incubation of samples with 3% triphenyl tetrazolium chloride at 30 ^oC, pH=7 and addition of 37% formaldehyde to form formazan for an hour. The optimal wavelength for formazan was measured between 500-700 nm.

Superoxide dismutase (SOD) activity was performed as described in the l,terature [39] which quantifies the inhibition of photochemical reduction of nitro blue tetrazolium chloride caused by SOD activity. SOD activity was monitored for 5 min with 1 min intervals at 560 nm.

Catalase activity (CAT) was measured by the reduction after the addition of phosphate buffer (pH=7.0) containing 0.07% H_2O_2, at 240 nm for 4 min [40].

3. Results and Discussion

To overcome the methodological issues and link the toxicity assay with environmental or test conditions, it is important to conduct appropriate sample pretreatment for ZnO NPs. For this purpose, three sample pretreatment methods were conducted using available studies as mentioned in Supplementary Table S1 and our previous study [2]. The method-I is mostly used both in toxicity and characterization studies, however, this method does not involve any sample pretreatment to reflect neither environmental nor test conditions. The method-II is frequently applied in toxicity studies; on the other hand, it can reflect only the test conditions, not the environmental matrix. The method-III reflects the environmental matrix. It is obviously that test conditions and environmental matrix can affect the surface properties of NPs. Moreover, NPs mostly interact with the medium components of seawater, soil or air [2,8-13]. The previous studies observed that surface properties were mostly influenced from medium components. Therefore, sample pretreatment is important step to reflect the environmental and control matrix. In addition, sample pretreatment plays an important role in the selection of controlled test conditions (control group) and comparison between controlled and environmental conditions. For this purposes, different sample pretreatment methods were applied to examine the effect on bacterial bioassay. The viability of the tested bacteria was shown in Figure 1. A slight or no inhibition (0 to < 10%) was observed on the gram-negative *E. coli* for the applied procedures. The limited inhibition can explain that the bacteria cell wall and ZnO NPs surface have the same charge. However, high inhibition (20-70 %) was shown against the other gram-negative bacteria,

P. aeruginosa. Depending on the composition of cell walls, *E. coli*, and *P. aeruginosa* can be exhibiting different interactions with ZnO NPs. *E. coli* had high viability compared to *P. aeruginosa*. This result can explain that the thicker disaccharide cell walls of *E. coli* compared to *P. aeruginosa* can limit the interaction with ZnO NPs. Furthermore, high inhibition (>50%) of selected gram-positive *S. aureus* was observed in all procedures, because of the difference between the cell wall charge of the *S. aureus* and surface charge of ZnO NPs which increase the interaction [2,8-11].

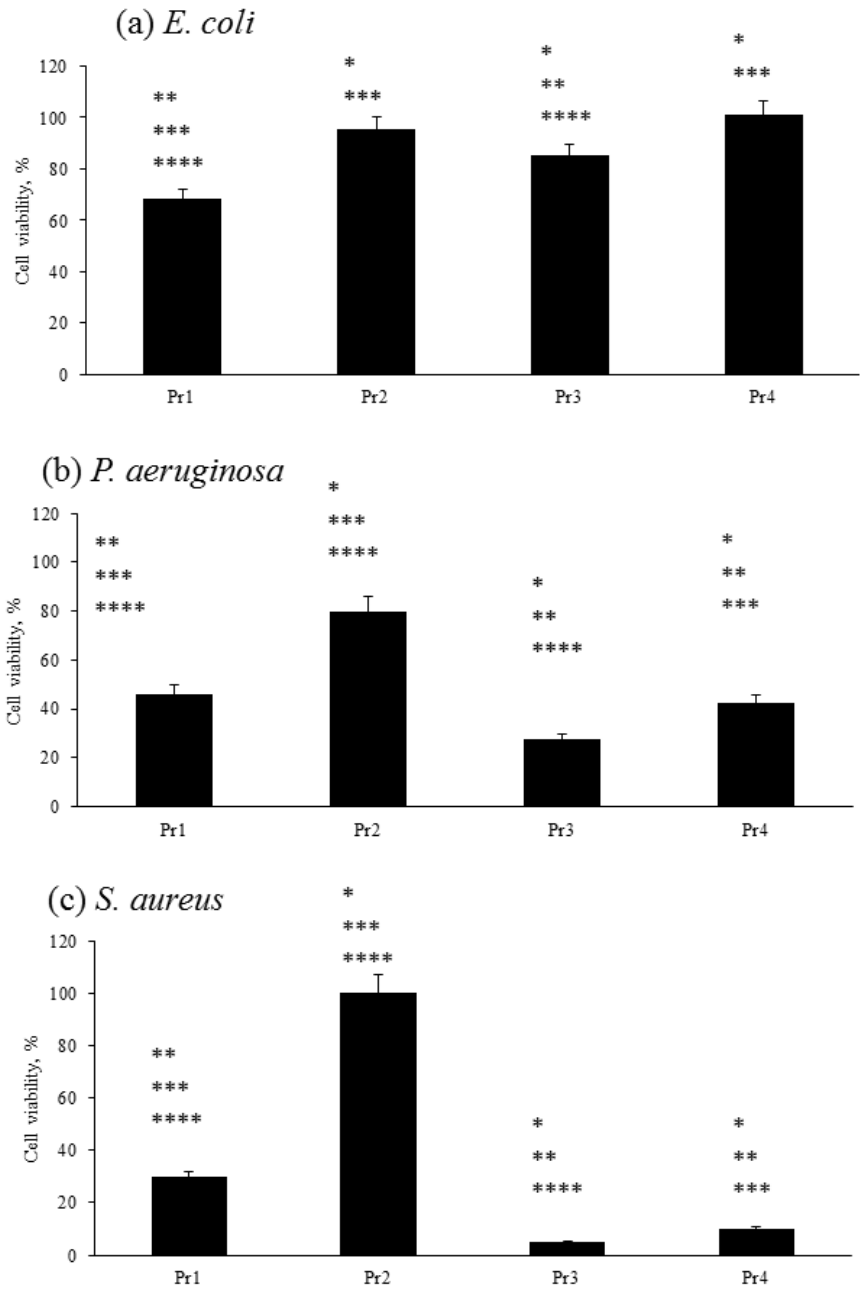
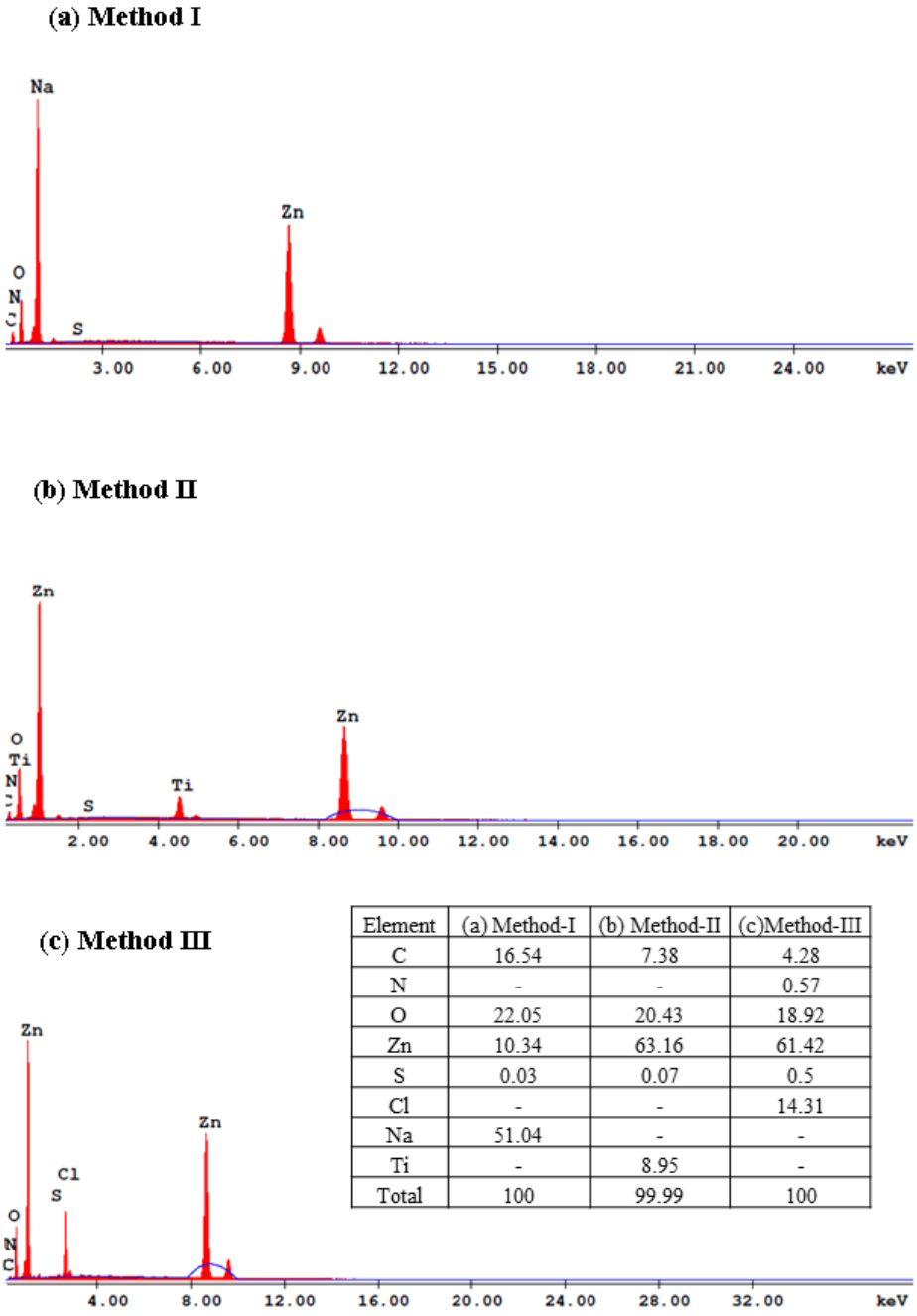


Figure 1. Cell viability percentages of tested bacteria exposed to ZnO NPs with applied procedure associated impact (a) viability of *E. coli*, (b) viability of *P. aeruginosa* , (c) viability of *S. aureus*

Pr1: Procedure1, Pr2: Procedure 2, Pr3: Procedure3, Pr4: Procedure 4. Different symbols for the bars indicate statistically significant results; ***** in relation to Procedure 1 ($p<0.05$). ****** in relation to Procedure 2 ($p<0.05$). ******* in relation to Procedure 3 ($p<0.05$). ******** in relation to Procedure 4.

The viability difference between bioassay procedures evaluated according to the Tukey test. There were significant differences between the applied procedures and this observation was free from the bacteria type. The viability order among procedures were obtained as Procedure4>Procedure2>Procedure3>Procedure1, Procedure2>Procedure1>Procedure4>Procedure3, and Procedure2>Procedure1>Procedure4>Procedure3 for *E. coli*, *P. aeruginosa* and *S. aureus*, respectively. Most sensitive bacteria were *S. aureus* and *P. aeruginosa* to the applied procedures. The results showed that high viability can be obtained using Procedure2 compared to other procedures for tested bacteria. Moreover, a high correlation was observed not only between Procedure 3 and Procedure 4 but also between *P. aeruginosa* and *S. aureus*.



Element	(a) Method-I	(b) Method-II	(c)Method-III
C	16.54	7.38	4.28
N	-	-	0.57
O	22.05	20.43	18.92
Zn	10.34	63.16	61.42
S	0.03	0.07	0.5
Cl	-	-	14.31
Na	51.04	-	-
Ti	-	8.95	-
Total	100	99.99	100

Figure 2. EDX spectrum of ZnO NPs treated with different sample pretreatment (a) Method-I, (b) Method-II, (c) Method-III. The table indicates the elemental composition according to sample pretreatment

The results also indicated that applied bioassay procedure has a great impact on the inhibition level and inhibited species. Because, sample pretreatment method and related bioassay procedure was different.

To evaluate the effect of sample pretreatment on the toxicity of ZnO NPs, it needs to reveal the inhibition pathway of the tested procedure. Because the inhibition pathways are detailed processes in which substances are either effective in the process or transformed into other substances. Detailed examination of the processes is important in many ways: (i) to control the process, (ii) to choose effective conditions, (iii) to find correlation between systems not otherwise obviously related, (iv) to permit unification and understanding of matter is of great importance in both theory and practice. Therefore inhibition pathways were examined using physicochemical and biochemical phenomena.

The chemical composition of the ZnO NPs recorded with the EDX system and is given in Figure 2. According to the EDX spectrums, the main elemental components of the ZnO NPs were changed with the applied sample pretreatment. The contents of C and O decrease with Method-II and Method-III compared to Method-I. Additionally, Cl and S ions were introduced to the surface of the ZnO NPs with the seawater treatment (Method-III). The decrease in the content of C and O can be explained that surface impurities can be cleaned by the treatment of ultra-pure water (Method-II) and seawater (Method-III). Contrarily, an increase in the Cl and S content with the treatment of Method-III can be linked to the sorption of the chemical compounds found in seawater (as shown in Supplementary Table S3).

The FTIR spectra of ZnO NPs obtained with sample pretreatment methods are shown in Figure 3. The main characteristic peaks of ZnO NPs treated with Method-I were observed at 650 cm^{-1} and 850 cm^{-1} (C-H), 1350 cm^{-1}, 1700 cm^{-1} and 2300 cm^{-1} (C=O) and a broad absorption band between 3100-3600 cm^{-1} (O-H) was obtained. The FTIR spectrum of ZnO NPs treated with Method-II shows signals at 1400 cm^{-1}, 1500 cm^{-1} and broad absorption band between 3100-3600 cm^{-1} mainly ascribed to C-H and O-H groups

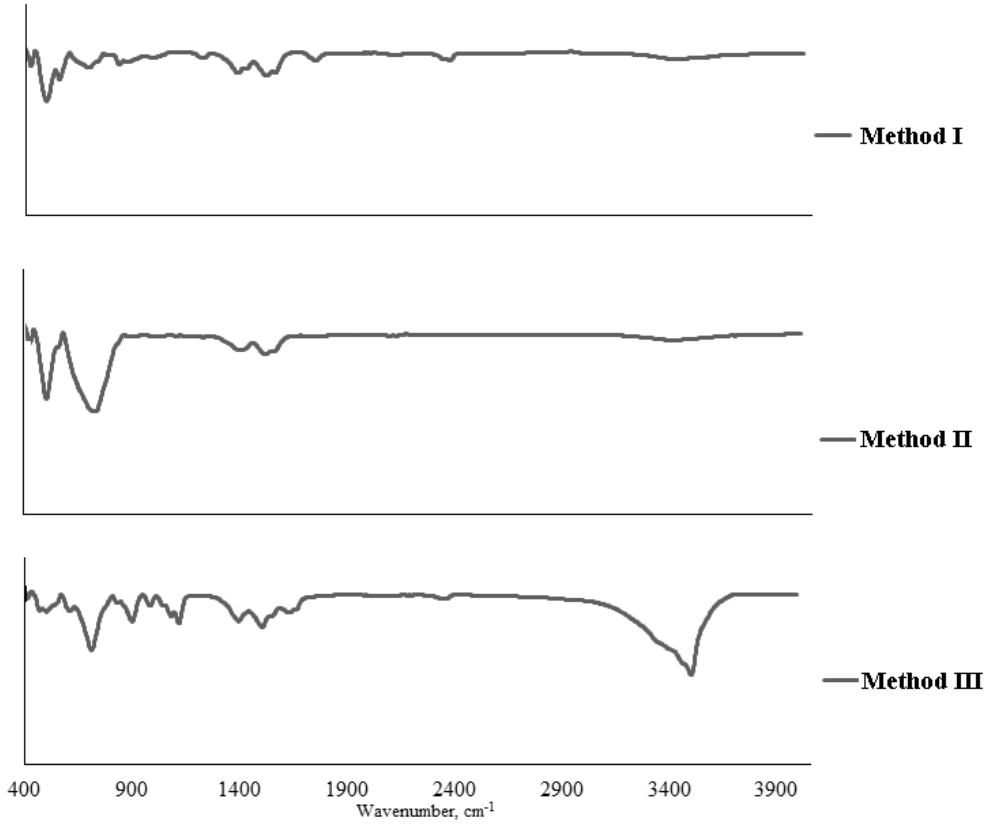


Figure 3. FTIR spectra of the ZnO NPs with the different sample pretreatment (**a**) Method-I, (**b**) Method-II, (**c**) Method-III

On the other hand, most of the C-H and C=O peaks seen in Method-I were not observed with Method-II. This observation collaborates with the EDX results. The FTIR spectrum of ZnO NPs treated with Method-III, the major peaks were observed at 700 and 900 cm^{-1} (C-Cl), 980 cm^{-1} (C=C), 1100 and 1600 cm^{-1} (C-N), 1380 cm^{-1} (S-O), 1500 cm^{-1} (N-O) and 3500 cm^{-1} (N-H). The formation of Cl-, N- and S- related groups are also in agreement with the EDX spectrum.

Table 2 shows the particle sizes and zeta potentials of ZnO NPs treated with different sample pretreatment methods. Despite original particle size of ZnO NPs was reported to be <50 nm by the manufacturer, the particle sizes were significantly different and found as 301±12, 671±33, and 548±21 nm for Method-I, Method-II, and Method-III, respectively. The significantly different particle sizes were obtained among the sample pretreatment methods. The zeta potentials were found as -29.5±1.5 mV, -14.2±0.6 mV, and -16.9±0.7 mV for Method-1, Method-II, and Method-III, respectively. The results showed that there was a significant difference between methods. As expected, the particle sizes had a highly negative correlation with their zeta potentials. It is known that chemistry of aquatic environment influences the formation of agglomerates and surface potentials due to deposition of organic materials, ionic strength, ion concentration and detachment of surface impurities [36,41-43].

Table 2. Particle size and zeta potentials of ZnO NPs (N:3, SD<5%)

Sample pre-treatment method	Particle size (d.nm)	Zeta potential (mV)
Method-I	301±12	-29.5±1.5
Method-II	671±33	-14.2±0.6
Method-III	548±21	-16.9±0.7

In order to investigate the effect of sample pretreatment on the particle morphology, SEM images were taken from ZnO NPs (Supplementary Figure S1). It was shown that agglomerates were slightly formed with the Method-II and Method-III compared to Method-I, which is in good agreement with the particle sizes and zeta potentials.

The EDX, SEM, DLS results indicated that attachment/detachment of chemical components, formation of new functional groups or changing the intensities of existed functional groups with the sample pretreatment methods reflect the matrix effect. Also, the functional groups on the surface are the key factors for the surface charge and formation of agglomerates. All these results showed that the main physicochemical properties (agglomeration, stability, functionality, composition etc.) were changed with the sample pretreatment. It means that new structural NPs were obtained with each sample pretreatment method, especially with the treatment of seawater. This is not unexpected results. On the other hand, the important point is how these changes affected the toxicity level and toxicity mechanism of NPs

One of the main inhibition mechanisms can be identified by chemical interactions between NPs and the components of the cell envelope. Antioxidant activity is probably the best-known indicator of the mentioned chemical interactions. Antioxidants play an important role in preventing or limiting the damage caused by reactive species. Also, DeH, SOD, and CAT are some of the prominent antioxidant enzymes that efficiently protect against the harmful biological events [44,45]. In order to understand the effect of applied procedures on the mechanism of the inhibition, antioxidant activity and some important antioxidant enzymes (DeH, SOD, CAT) were examined. Since CAT responses were not significantly changed compared to control, antioxidant, DeH and SOD responses were evaluated.

Antioxidant activity was affected by the applied procedures and the results are independent of the inhibition levels and bacteria species (Figure 4). High antioxidant activities were obtained in Procedure 3 and Procedure 4 compared to Procedure 1 and Procedure 2. For the tested bacteria, there were no differences between Procedure 1 and Procedure 2. Likewise, there were no differences between Procedure 3 and Procedure 4. However, there were significant differences between Procedure 1-2 and Procedure 3-4.

As shown in Figure 4(aII - cII), SOD activity of *E. coli* decreased at the level of 16-28% in all procedures, whereas slight decreases were obtained on SOD activities of *S. aureus* in all procedures (6-19%). SOD of *P. aeruginosa* was also decreased in Procedure 1 (18%) and Procedure 2 (25%). Lower SOD responses were obtained with Procedure 1 and 2 for all bacteria compared to other procedures.

Figure 4 (aIII-cIII) depicts the DeH activities of tested bacteria. DeH activity of *E. coli* had negative responses in Procedure 3 (32%) and Procedure 4 (41%), while there was no significant change in other procedures. DeH activity of *P. aeruginosa* decreased at the level of 20%, 32%, and 35% in Procedure 2, 3 and 4, respectively. The highest negative responses were obtained for DeH activity of *S. aureus* in Procedure 3 (61%) and Procedure 4 (62%); slight negative response (19%) and no change were observed in Procedure 1 and in Procedure 2, respectively.

In order to evaluate the physicochemical and biochemical phenomena on the toxicity, the inhibition dependency was summarized and shown in Figure 5. Interaction mechanisms observed for Procedure 1 with *E. coli*, *P. aeruginosa*, and *S. aureus* are reduced particle size+SOD, reduced particle size+SOD+DeH, and reduced particle size+SOD+DeH, respectively. On the other hand, Procedure 2, 3 and 4 affected all tested bacteria by the same pathway which is reduced particle size+SOD, agglomeration+DeH, and surface chemistry+DeH, respectively. This observation does not depend on the bacteria type. Likewise, each toxicity procedures have different inhibition levels and inhibition pathways. Obtained results indicate that sample pretreatment methods and their toxicity procedures determine the pathway of the inhibition. Moreover, the measurement quality which includes reproducibility and repeatability has impact on the selection of method [2]. Therefore, we also discussed the standard deviations of the physicochemical parameters. The results indicated that there were no specific differences between standard deviations of the sample pretreatment methods for the tested parameters. This result was different with our previous study which is related to titanium nanoparticles [2]. In the previous study, Method-III had highest precision compared to other methods, and the order of the precision of tested NPs is Method-III > Method-I > Method-II. This observation showed that sample pretreatment might be specific for each nanoparticle.

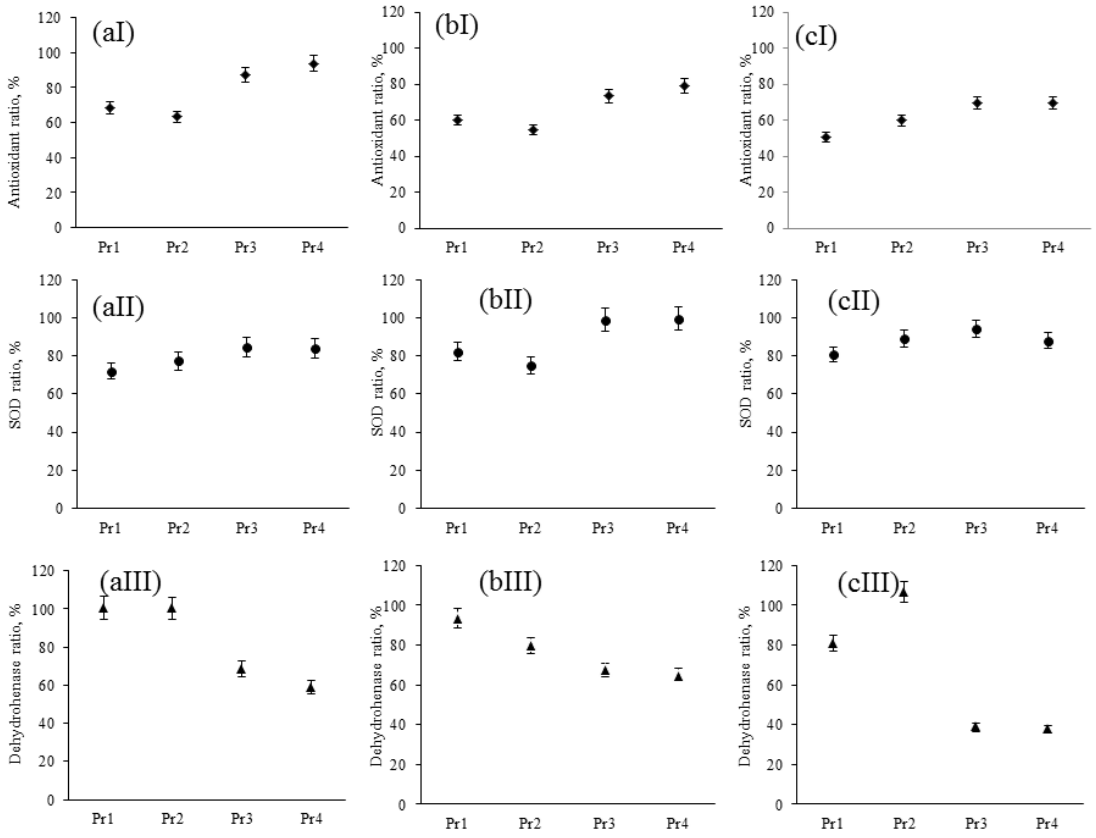


Figure 4. Results of antioxidant capacity, SOD and DeH of tested bacteria exposed to ZnO NPs with applied procedure associated impact (**a**) *E. coli*, (**b**) *P. aeruginosa* , (**c**) *S. aureus.* I. Antioxidant activity, II. SOD activity, III. DeH activity

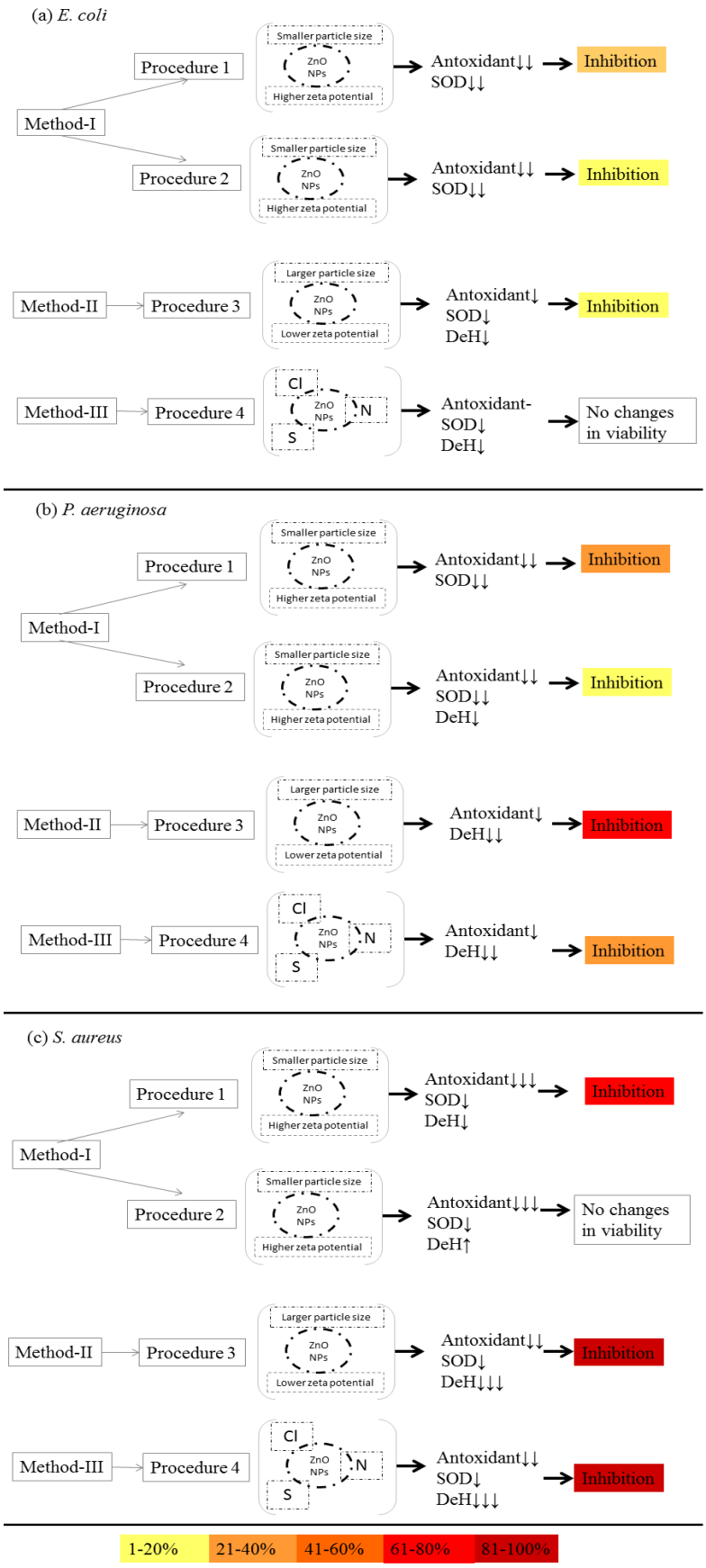


Figure 5. Scheme of the effective pathway of the ZnO NPs on the bacteria according to the different sample pretreatment methods.

These results can be helpful for the development of the analytical procedure for the ecological risk assessment of ZnO NPs through physicochemical and toxicological aspects. Using the results respecting precision of bioassay, control experiment, two methods can be developed and optimized, which are;

I. Ecotoxicity Assessment

Bacteria type: *P. aeruginosa*, and *S. aureus*,
Sample pretreatment method: Method-I1
Bioassay procedure: Procedure 2
Control experiment: Sample pretreatment method: Method-I, Bioassay Procedure: Procedure 1

II. Ecotoxicity Assessment

Bacteria type: *P. aeruginosa*, and *S. aureus*,
Sample pretreatment method: Method-III
Bioassay procedure: Procedure 4
Control experiment: Sample pretreatment method: Method-II, Bioassay Procedure: Procedure 3.

Method-I allows examining both environmental and control conditions in the presence of the ZnO NPs with bacteria bioassay. Bioassay Procedure1 combined with the Method-I allow to investigate control conditions, and Bioassay Procedure2 combined with the Method-I reflect environmental conditions. However, the surface chemistry of NPs may be interacted with the growth medium components, and the contribution of this interaction may not be measured. Method-III using Bioassay Procedure4 clearly reflects the environmental conditions both on the NPs and selected species during the whole procedure. Method-II using Bioassay Procedure3 can be used to reflect the pure control condition and as a control of the Method-III combined with Bioassay Procedure4. On the hand, this kind of bioassay and sample pretreatment methods may limit the investigation of aging effect on NPs surface chemistry.

4. Conclusion

We demonstrated three sample preparation methods to link the toxicity procedures and physicochemical characterization. The results showed that toxicity levels and mechanisms were changed by the applied sample preparation, and the results are mostly independent of the bacteria type. These results explain how sample pretreatment influences the interaction of ZnO NPs with the microorganism.

To our knowledge, this is one of the rare study which evaluates the effect of sample pretreatment on the toxicity and physicochemistry of ZnO NPs. The study can be useful to develop standardized ecotoxicity procedures for ZnO NPs using bacterial bioassay. In conclusion, to make a realistic ecotoxicity assessment of ZnO NPs, selection of sample pretreatment method that is consistent with both particle characterization and toxicity bioassay is important.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/journal-of-chemical-metrology

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