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Investigating Toxicity and Antibacterial Aspects of Nano ZnO, TiO_2 and CuO with Four Bacterial Species

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ABSTRACT

This study investigates the toxicity and antibacterial aspect of nano ZnO, TiO_2, and CuO with bacterium species bioassay.
The stock nanoparticles (NPs) suspensions (100mmol/L) were diluted with Mueller-Hinton Agar medium to make a series of media containing 0.05-75mmol/L NPs. The refined bacteria cultured on these media and growth inhibition of bacteria determined. The obtained data was analyzed using Probit analysis in SPSS software and LC_{50} as toxicity and 100% mortality as antibacterial characteristic of NPs was determined.
The LC_{50} of nano ZnO was lesser than TiO_2 and CuO. It was 2.13, 7.87, 1.06, and 11.28mmol/L for *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa* respectively. Also the LC_{50} of nano TiO_2 for these bacteria was 68.47, 34.43, 1.08, and 29.51 and for nano CuO it was 29.84, 20.63, 11.76, and 16.01mmol/L respectively. The NOEC analysis showed that for all three NPs the 0.05mmol/L is quite safe for all four bacteria. As antibacterial characteristic of NPs, the 100% mortality analysis showed that in 34.03mmol/L of Nano ZnO, 190.74mmol/L of Nano TiO_2 and 84.41mmol/L of Nano CuO all used bacteria will be killed.
This investigation suggests that *S. aureus*, due to its high sensitivity to all three NPs, could potentially be used as an effective indicator bacterium in the NPs toxicity bioassay. Comparing NPs LC_{50}s, nano TiO_2 can be suggested as an alternative instead of nano ZnO where it is practicable. Selective toxicity of NPs to different bacteria species demonstrated that NPs can be manipulated for biomedical, antibacterial or health applications.
KEYWORD: Disinfection, Toxicity, NP, LC_{50}, Bacterial indicator, Nanotoxicology.

1. INTRODUCTION

Nowadays more than 500 consumer products in the markets are related to nanotechnology (Jones and Grainger 2009). Nanoscale titanium dioxide (TiO_2), zinc oxide (ZnO), and copper oxide (CuO) are common nanoparticles (NPs) with a variety of applications. TiO_2 is one of the best opacifiers and is used as a pigment in paints, inks, paper, sunscreens, cosmetics, and plastics (Adams et al., 2006; Long et al., 2006). ZnO acts as both pigment and semiconductor in some industries (Adams et al., 2006). TiO_2 and ZnO NPs are already produced in industrial scale (The royal society 2004). CuO is used in wood preservation and antimicrobial textiles (Cox 1991; Gabbay et al., 2006).

Even if the potential benefits of nanomaterials (NMs) are unexceptionable, wide application of them results in the use of millions of tons of NPs as basic material, leading to the introduction of NPs into the environment, food, and water resources (Jones and Grainger 2009). Progressive use of NPs in the environment requires a fundamental understanding of their mode and range of toxicity. There is some information available about the possible risks associated with NMs including the risks for environment and human beings (Bystrzejewska Piotrowska et al., 2009; Barbu et al., 2009; Lockman et al., 2003; Naddafi et al., 2011).

Recently, some studies showed that exposure to ZnO NPs can result in oxidative damage and inflammation in vascular and lung endothelial cells (Gojova et al., 2007; Lin et al., 2009). Other studies have shown that NP of some metal oxides, can be toxic to human cells (Long et al., 2006; Nel et al., 2006) crustaceans (Lovern et al., 2006; Zhu et al., 2009) algae (Aruoja et al., 2009; Wang et al., 2008), and fish (Handy and Shaw 2007; Federici et al., 2007) and

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have growth inhibition characteristics in prokaryotic cells (Brayner et al., 2006; Thill et al., 2006). Most studies that investigated the metal oxide NP toxicity have focused on mammalian cells, primarily on transformed cancer cell lines; however, NPs effects on bacterial systems have not been investigated except in a few recent studies. Brayner et al. (2006) reported the toxicity of ZnO NPs on just one prokaryotic. They reported significant growth inhibition of *Escherichia coli* at concentrations more than 3mmol. Block et al. (1997) concluded that TiO_2 enhances sunlight for killing bacteria in aqueous solution and reported a concentration of 0.01% TiO_2 as the best concentration for killing bacteria and tenfold concentration (lower or higher) were successively less effective.

ZnO has been reported to have antibacterial growth inhibition traits, with *Bacillus subtilis* being more sensitive to its effects than the *E. coli* (Sawai et al., 1995). The minimal inhibitory concentrations were from 2000-12,500ppm for *Bacillus subtilis* and 50,000- 100,000ppm for *E. coli* depending on particle size (Sawai et al., 1996).

Although few investigations on bacteria have been conducted in aqueous media, based on previous studies, NPs acquire agglomeration properties as a result of mixing up and contact with each other, therefore their diameter increase (Navarro et al., 2008; Baveye and Laba 2008). Besides, the diameter of NPs may affect the level of their arrival into cells and thus their toxicity (Baveye and Laba 2008; Sawai et al., 1996). Hence in previous studies, the effect of dispersion of system that can lead to NPs slowly settle down and agglomeration in the aqueous media was ignored. It can result in toxicity concentration reports lower than actual toxicity concentration. So the toxicity of NPs in solid environments is largely unknown, while the soil and solids are believed to be the ultimate sinks for NMs in the environment (Klaine et al., 2008). The study of NPs in the solid phase can help the clarification of NPs toxicity mechanisms.

On the other hand, comparison of previous studies on the toxicity of NPs with the same bioassay creature show that they can be very different in results because of individual and genetic differences of creatures as well as differences in chemical conditions of culturing media, NPs' characteristics and NPs preparation methods (Adams et al., 2006; Naddafi et al., 2011). Therefore, for the best comparison of results, it is suggested that all effective parameter such as creature or NP types be investigated in one research. Although, many studies have been conducted almost on one bacteria species or one NP type and in aqueous media (Adams et al., 2006; Liu et al., 2009; Brayner et al., 2006; Reddy et al., 2007). Furthermore, previous studies did not determine the EC_{50}, so comparison of data of different studies is difficult. Hence this is the first comprehensive study that investigates three NPs EC_{50}s with four bacteria in the solid media. Also, determining the 100% mortality in this study can result in highlighting the NPs antibacterial properties. Compared with the organic materials inorganic antibacterial compounds (like NPs) are safer and more stable at high temperatures and pressures (Liu et al., 2009), so our results can lead to a variety of novel applications ranging from anti-bacterial to biomedical and therapeutic treatments.

2. MATERIAL AND METHODS

The NPs characteristics are given in Table 1. The stock suspension of the NPs were prepared just before each test and placed in a sonicator for 30 minutes. At least 5 different concentrations of desired solution are needed to determine the LC_{50}s (APHA and AWWA 2005). Considering the results of other studies and for increasing the precision of the results, the mortality was tested at 14 concentrations in the range of 0.05 to 75mmol/L. The stock NPs suspension (100mmol/L) was diluted with Mueller-Hinton Agar (MHA) medium to make a series of media containing NPs with concentration of 0.05-75mmol/L. For inducing the sterilization condition, the media were then autoclaved. NP-induced toxicity tests on bacteria were conducted in these media.

Table 1. The characteristic of used NPs.

NP	Supplier company	Purity %	Size (nm)	Specific surface
Nano-TiO_2	Merck	99	20	40
Nano-ZnO	Merck	99	20	90
Nano-CuO	Merck	98	60	80

To evaluate the applicability aspects of NPs such as antibacterial properties in solid media, bacterial species were obtained from the municipal sludge wastewater treatment plant of Kerman (Iran). For this aim the sludge samples cultured on the nutrient agar medium. This media incubated for colony forming for 24-48 hr at 37ºC and then bacteria species were isolated in a certified laboratory. After culturing the isolated bacterial species, NP toxicity tests were performed by inoculating bacterial cells on MHA plates containing different concentrations of NPs (0.05-75mmol/L) and without NPs (as a control). The inoculated cells were estimated to be 200 CFU per plate, and all measurements were performed in triplicate and mean value of mortality in comparison with the controls were determined.

The mean of mortality rate for triplicate experiments were analyzed using Probit analysis, and LC_{50}s were determined. The results obtained from Probit analysis were used to plot diagrams in Microsoft Excel 2007. No observed effect concentration (NOEC) was also determined by Probit analysis, via calculation of concentration in which the mortality rate was 10%. In addition, the 100% mortality rate was determined by calculating the concentration in which the mortality rate was 99%, using Probit analysis.

3. RESULTS AND DISCUSSION

The results of Probit analysis (Figure 1-3) showed a concentration dependent toxicity for *bacteria* (in all cases Pearson correlation coefficient more than 0.96 and $P < 0.05$). Mortality rate diagrams showed a parallel change in mortality rate of *P. aeruginosa* and *B. subtilis.* But *E. coli* and *S. aureus* have non-parallel mortality rate diagrams when exposed to the nano ZnO (Figure 1) and nano TiO_2 (Figure 2). The parallel pattern may be related to the similar mechanism of toxicity. The main mechanism of toxicity of NPs is thought to be through oxidative stress (OS) (Kohen and Nyska 2002).

Figure 1. Mortality rate for *bacteria* at the various concentrations of nano ZnO

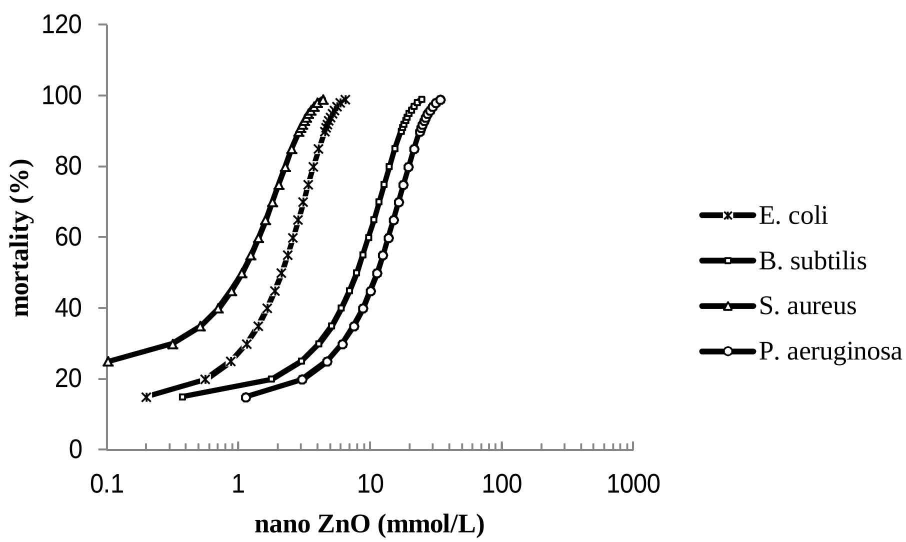


Figure 2. Mortality rate for *bacteria* at the various concentrations of nano TiO_2

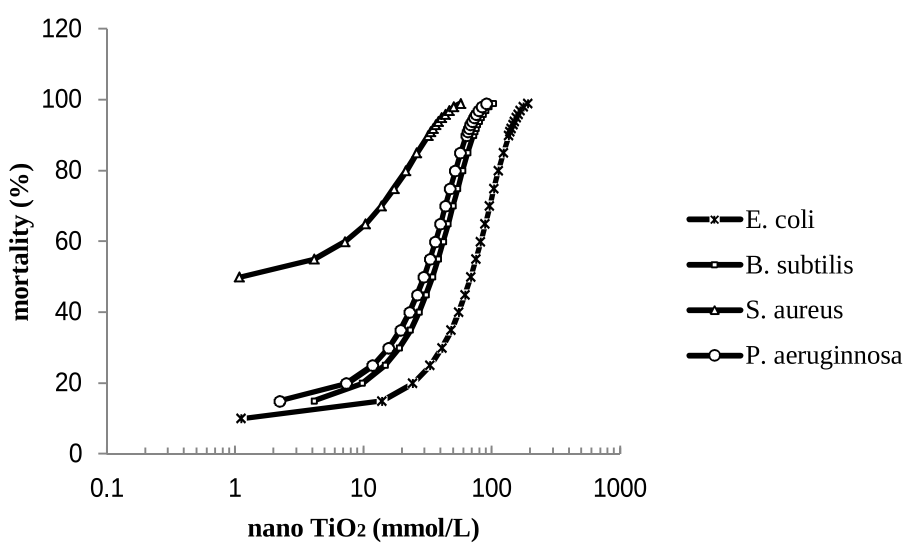
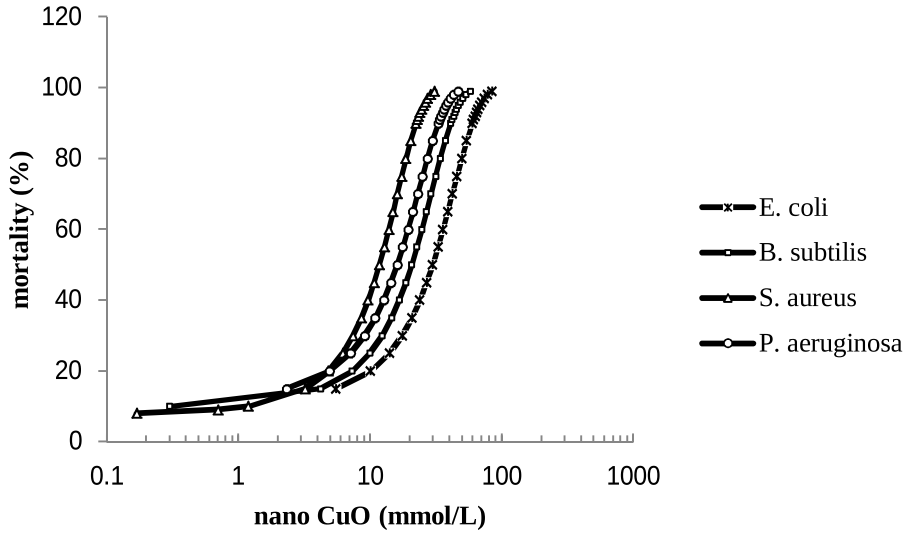


Figure 3. Mortality rate for *bacteria* at the various concentrations of nano CuO



The LC_{50}s of nano ZnO, nano TiO_2 and nano CuO have been illustrated in Tables 2-4. According to these tables and Figure 1-3, nano ZnO is the most toxic NP and its LC_{50} is 2.13, 7.87, 1.06, and 11.28mmol/L for *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa*, respectively. Also nano CuO is more toxic than nano TiO_2 except in the case of *S. aureus* that nano TiO_2 is more toxic than nano CuO (Tables 3 and 4). So, because some of the applications of nano ZnO and TiO_2 are similar, the application of nano TiO_2 instead of nano ZnO is suggested to be considered and studied.

Table 2. LC_{50}, NOEC and 100% mortality (mmol/L) of nano ZnO

Bacteria	Nano ZnO LC_{50} (mmol/L)	95 % confidence intervals for LC_{50}		NOEC (mmol/L)	100 % mortality (mmol/L)
		Lower bound	Upper bound		
E. coli	2.13	1.64	2.76	0.00	6.47
B. subtilis	7.87	6.42	9.38	0.00	24.70
S. aureus	1.06	0.46	1.80	0.00	4.37
P. aeruginosa	11.28	8.66	14.24	0.00	34.03

Table 3. LC_{50}, NOEC and 100% mortality (mmol/L) of nano TiO_2

Bacteria	Nano TiO_2 LC_{50} (mmol/L)	95 % confidence intervals for LC_{50}		NOEC (mmol/L)	100 % mortality (mmol/L)
		Lower bound	Upper bound		
E. coli	68.47	52.68	105.31	1.12	190.74
B. subtilis	34.43	28.10	42.68	0.00	102.44
S. aureus	1.08	-73.93	13.04	0.00	57.10
P. aeruginosa	29.51	24.25	35.94	0.00	90.76

Table 4. LC_{50}, NOEC and 100% mortality (mmol/L) of nano CuO

Bacteria	Nano CuO LC_{50} (mmol/L)	95 % confidence intervals for LC_{50}		NOEC (mmol/L)	100 % mortality (mmol/L)
		Lower bound	Upper bound		
E. coli	29.84	24.87	35.93	0.00	84.41
B. subtilis	20.63	17.69	24.20	0.30	57.54
S. aureus	11.76	10.56	13.08	1.19	30.95
P. aeruginosa	16.01	12.56	20.30	0.00	46.77

No similar study has determined the LC_{50}, 100% mortality or NOEC of these NPs in solid media and no complete comparison can be made. But a study supports our results by reporting nano ZnO water suspensions more toxic than other NPs and TiO_2 as having the lowest toxic level (Adams et al., 2006).

Results of 100% mortality showed all tested bacteria species at a minimum concentration of 34.03mmol/L ZnO get perished and in antibacterial applications of NPs, nano ZnO is more effective than two other tested NPs. The 100% mortality of all tested bacteria species for TiO_2 and CuO was at 190.74 and 84.41mmol/L, respectively. According to the results, at 0.05mmol/L of all NPs no mortality occurred for all tested bacteria species (results are not shown). In this regard, for nano TiO_2 and CuO, the mortality rate was less than 10% in a concentration of 0.1mmol/L of these

NPs. But according to the result of Probit analysis that have been shown in Tables 2-4, the NOEC for the most cases is 0.00mmol/L.

The results showed *S. aureus* is the most sensitive bacteria against tested NPs in comparison with 3 other bacteria. As in the bioassay tests, the best reliability and validity is obtained through using the most sensitive creatures, *S. aureus* can be offered in the NP toxicity tests as a good candidate as indicator. Unlike *S. aureus*, the *P. aeruginosa* is the least sensitive bacteria against ZnO and *E. coli* is the least sensitive bacteria against TiO_2 and CuO. Hence it can be concluded that, these bacteria can not be a proper indicator for determining the environmental toxicity of these NPs. regarding the antibacterial applications, these NPs are not suitable choice for disinfection against *E. coli* and *B. subtilis*. The results of our study suggest *S. aureus*, as a gram positive bacterium, is a sensitive species against the tested NPs and this finding is supported by Adams et al. (2006), who studied eco-toxicity of nanoscale TiO_2, SiO_2, and ZnO water suspensions. They tested *B. subtilis* and *E. coli* in their study and reported that gram positive bacterium is more sensitive in comparison with gram negative. But generalization of this conclusion is not always valid, because although in Adams study the gram positive *B. subtilis* was more sensitive against nano ZnO, in our study gram negative E. coli was more susceptible (in comparison with *B. subtilis*) when it was exposed to nano ZnO. Also the concentrations of ZnO required killing 50% of E. coli bacteria were smaller than the concentrations which were published previously (Adams et al, 2006). Adams et al reported 48% growth inhibition occurs in concentration of 1000ppm. Such a difference may be due to genetic and individual differences of bacteria as well as differences in chemical conditions of *bacteria*'s culturing media and NPs' characteristics (size, shape or density) (Adams et al, 2006). An obvious difference between Adams and our study situations is that, in our study, agar media (solid media) instead of broth (liquid media) was used for bioassay. In our study we chose agar media because if broth is used part of NPs may precipitate through agglomeration in the broth media and make bacteria exposure concentrations less than expected (Liu et al, 2009). Furthermore, using the solid media in the bioassay, results in more practicable data for application of NPs as a sludge disinfectant.

The LC_{50} of TiO_2 is illustrated in Table 2. According to this table the LC_{50} of TiO_2 for E. coli is nearly 32 times more than that of *B. subtilis* but comparison of 100% mortality shows that this difference is less than 2 times. This finding insists that reporting solely the LC_{50} of NPs doesn't give a correct imaging of a NP's toxicological characteristics, especially when the NPs are considered as antibacterial, and along with LC_{50}, 100% mortality rate should be regarded.

4. Conclusion

According to the results of this investigation, different bacteria species exhibit various toxicological responses to different NPs. This selective trait of NPs suggests that they can be manipulated for biomedical and antibacterial applications or agricultural product preservation. For this aims, there is a need for more research on sensitivity of other bacteria on the different NPs.

Nano ZnO exhibited the best antibacterial properties against four tested bacteria than TiO_2 and CuO and its toxicity, as indicated by LC_{50}, increased from *P. aeruginosa* to *B. subtilis* to *E. coli* to *S. aureus*. This sequence is some different for TiO_2 and CuO but for all three NPs *S. aureus* is the most sensitive bacterium. Accordingly this investigation suggests that *S. aureus* can potentially be used as an effective indicator bacterium in the NPs toxicity bioassay.

Comparing the LC_{50} of nano ZnO and nano TiO_2 suggests that, where possible, the less toxic nano TiO_2 should be used instead of nano ZnO.

This research revealed that the NPs can display selective toxicity to different bacteria. This property can be controlled for biomedical/antibacterial applications or agricultural preservation usage of NPs. It means despite the potential toxicity of some NPs, their unique properties can be manipulated for controlling the harmful microorganisms.

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