The use of antibacterial activity of ZnO nanoparticles in the treatment of municipal wastewater

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ABSTRACT

Nanotechnology holds great potential in advanced water and wastewater treatment to improve treatment efficiency. Zinc oxide nanoparticles (ZnO NPs) have received considerable attention due to their unique antibacterial activities toward various microorganisms that are commonly found in the environment. In the present study, ZnO NPs were synthesized through both mechano-chemical and sol–gel methods. The synthesized ZnO NPs were characterized through X-ray diffraction and transmission electron microscopy techniques. Then, their antibacterial activities against separated wastewater bacteria were evaluated by determining the zone inhibitor, the minimum inhibitory concentration, and the minimum bactericidal concentration. The results were compared with those obtained from wastewater after chlorine disinfection and ultraviolet (UV) disinfection. These studies demonstrated that the antibacterial activity of ZnO NPs depends on the type and the strain of bacteria. They have also demonstrated that the activity increases as the concentration of ZnO NPs increases. Overall, the experimental results suggest that ZnO NPs can potentially be an antibacterial reagent to treat wastewater. They can particularly be applied as a complementary method with UV disinfection. Thus, they can be developed as antibacterial agents to improve wastewater quality.

Key words | antibacterial, chlorine, disinfection, UV, wastewater, ZnO nanoparticle

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INTRODUCTION

The growth of population and human civilization forces the world's scientists to look for efficient ways to treat water and wastewater. Some physical, chemical, and biological methods can be applied for the disinfection of water and wastewater. Since the late 1940s, chlorination has been considered to have a major role in inactivating waterborne pathogens in many countries. However, many halogenated disinfection by-products (DBPs) have been characterized during chlorination (Zhang *et al.* 2009a, 2009b), which have been shown to be mutagenic, and can increase the risk of cancer (Monarca *et al.* 2004). Also, chlorine residuals are toxic to marine life (Ward & DeGraeve 1978). An effective alternative to chlorination is the disinfection of water and wastewater through ultraviolet (UV) irradiation to inactivate a variety of microorganisms (Chang *et al.* 1985; Das 2001; Bergmann *et al.* 2002; Hijnen *et al.* 2006). However, the germicidal efficiency of UV irradiation strongly depends on the total suspended solids in effluent, which will reduce germicidal efficiency or UV effect (Chang *et al.* 1985), although low-pressure UV produces undesirable DBPs (Bergmann *et al.* 2002; Hijnen *et al.* 2006). Despite the fact that municipal wastewater contains a variety of pathogenic organisms of human origin, it is used for the irrigation of plants. Therefore, it is highly necessary to come up with an advanced method of disinfection to reuse the municipal wastewater in agriculture (Mujeriego & Asano 1999).

To overcome the limitations of traditional disinfection methods, research has focused on developing alternative ones (Qu *et al.* 2013). Many nanomaterials, such as nano-Ag, nano-ZnO, nano-TiO_2, and carbon nanotubes have the potential to be used in the disinfection of water and wastewater (Li *et al.* 2008; Vahabi *et al.* 2011; Baruah *et al.* 2012). They display antimicrobial properties without strong oxidation. Therefore, they have a lower tendency

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to form DBPs (Qu *et al.* 2013). Among them, ZnO nanoparticles (NPs) are of particular interest due to their extensive application as an antimicrobial agent on a wide range of microorganisms. Previous studies revealed that ZnO NPs have bactericidal activities on both Gram-positive and Gram-negative bacteria (Adams *et al.* 2006b; Jones *et al.* 2008; Li *et al.* 2008; Liu *et al.* 2009; Zhang *et al.* 2012). It has been suggested that the antibacterial mechanism of ZnO NPs is due to the electrostatic interaction between the nanoparticles and the bacterial membrane (Zhang *et al.* 2007). The interaction between H_2O_2 and membrane proteins is also regarded as another proposed mechanism for the bactericidal properties of ZnO (Zhang *et al.* 2010, 2013). The relationship between the size of ZnO NPs and the antibacterial activity is ambiguous. Some reports indicated that disinfection behavior increases through reducing the size of ZnO NPs (Jones *et al.* 2008; Nair *et al.* 2009). Furthermore, it was found that a higher concentration of smaller particles with a higher surface area gives better antibacterial activity (Makhluf *et al.* 2005; Zhang *et al.* 2010). However, Franklin *et al.* did not observe any relationship between the size of ZnO NPs and the antibacterial activity (Franklin *et al.* 2007).

In this study, the synthesized ZnO NPs were produced through mechano-chemical and sol–gel methods. Then, they were characterized and tested for their antibacterial activity against *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus*, and *Staphylococcus epidermidis* bacteria in municipal wastewater collected from two areas in Mazandaran, Iran. The bactericidal activity of ZnO NPs was also compared with the conventional disinfection methods, i.e., chlorine and UV disinfection, which are commonly used for wastewater treatment in these two areas. In addition, the relationship between the concentration of ZnO NPs and the antibacterial activity was investigated in this study.

MATERIALS AND METHODS

Synthesis of ZnO NPs

Mechano-chemical method

Zinc acetate was dried at 120 °C in air for 24 h prior to its use. Zinc acetate and oxalic acid were mixed in an agate mortar with molar ratio of 2:3 and were powdered for 45 min at room temperature. The white precursor was calcined at 450 °C in air in a porcelain crucible for 30 min to prepare the ZnO NPs. The X-ray diffraction (XRD) pattern and the transmission electron microscopy (TEM) micrograph of the ZnO NPs are shown in the study by Alinezhad *et al.* (2012). The average crystallite size of the ZnO NPs was calculated by XRD line broadening technique using the Scherrer equation (Cullity 1978).

Sol–gel method

Zinc acetate (0.1 M) in ethanolic solution was refluxed for 180 min at 80 °C. Then, 0.2 L of the obtained reaction product (precursor) was diluted to 0.5 L with absolute ethanol. Lithium hydroxide powder was added to the precursor to give a final lithium concentration of 0.14 M. The mixture was then hydrolyzed in an ultrasonic bath. The hydrolysis reaction was continued at room temperature for about 1 h. The ZnO colloidal suspension was then filtered through a 0.1-pm glass fiber filter to remove dust and any undissolved lithium hydroxide. The average size of the ZnO NPs was estimated by XRD pattern. The scanning electron microscopy (SEM) micrograph of the ZnO NPs was recorded.

Municipal wastewater collection

The wastewater samples were collected from two different wastewater centers in Mazandaran (Ghaemshahr and Babolsar), Iran. The chlorine disinfection is applied in Ghaemshahr, station I, and the UV disinfection is used in Babolsar, station II. In both stations, large particles are removed through settling, flocculation, and sedimentation. In station I, chlorine is added to municipal wastewater at 1 kg d^{-1}. The remaining chlorine in the effluent is about 0.4–0.6 ppm. In station II, 32 low-pressure lamps emit essentially monochromatic light at a wavelength of 254 nm and 12 μWs cm^{-2} doses of UV. The effluent is reused in treatment plants. The wastewater samples were taken in 1 L glass bottles just before and after being disinfected by chlorine or UV, and were immediately transferred to the laboratory. Main chemical characteristics of the wastewater from both stations are shown in Table 1.

Bacterial separation

The wastewater samples (before and after being disinfected by chlorine or UV) were cultured on nutrient agar medium. After being incubated at 37 °C for 24–48 h, one loop from each colony was transferred evenly on EMB (eosin–methylene blue) and blood agar medium through a sterile glass rod. After being incubated at 37 °C for 24 h,

Table 1 | Main chemical characteristics of the wastewater

	Station I		Station II	
Main chemical characteristics of the wastewater	Input (ppm)	Output (ppm)	Input (ppm)	Output (ppm)
Chemical oxygen demand	326	32	150	20
Biochemical oxygen demand	233	14	75	10
Total dissolved solids	–	–	–	1,000
Total suspended solids	–	13	–	30–40

(–) The data are not available.

Gram-negative bacteria were characterized by an api-20A kit and Gram-positive bacteria were recognized by a biochemical test.

Antibacterial activity of ZnO NPs

Disk diffusion assay

ZnO NPs (prepared from two methods) were sterilized through UV light. Then, they were suspended in sterile normal saline and stirred until a uniform colloidal suspension was formed, after which 1.5×10^8 CFU mL^{-1} of each bacterium (*K. pneumoniae*, *E. coli*, *Proteus*, and *S. epidermidis* separated from each station) were sprayed on Müeller–Hinton medium. After letting the bacteria dry (within 5–10 min), 20 μL of nano-ZnO suspensions, whose concentrations varied from 0.01 to 50 mM, were dropped into a disk of 5 mm in diameter. The pure solvent was also used for control. The zone of inhibition was measured after the incubation process at 37 °C for 24 h. This experiment was repeated three times and the average number was calculated.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration which can cause complete growth inhibition. The broth microdilution method was applied to evaluate the MIC (Wiegand *et al.* 2008). One hundred microlitres of Müeller–Hinton broth, containing 10^5 CFU mL^{-1} of each bacterium, was added to each well. One hundred microlitres of ZnO suspensions (concentrations varied from 0.01 to 50 mM) was also poured into 96 sterilized well microplates and then incubated at 37 °C for 24 h. The first well, which was completely transparent and had no bacterial growth, was considered for MIC. Each concentration was tested three times and the results were averaged.

Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) is the lowest concentration at which the bacteria do not grow when they are transferred to fresh agar plate after the incubation period (99.9% killed). Ten microlitres of the content without turbidity wells was cultivated on the Müeller–Hinton plate. The number of colonies was counted after 24 h at 37 °C. The first well, which had three or fewer colonies, was regarded as MBC.

RESULTS

Characterization of ZnO NPs

Several methods are used to synthesize ZnO NPs. Some of these methods are sol–gel (Ristic *et al.* 2005), two step mechano-chemical thermal synthesis (Rajesh *et al.* 2012), precipitation method (Kumar *et al.* 2013), and microwave (Khoza *et al.* 2012). However, in most of these techniques, the high surface energy of nanoparticles causes them to agglomerate. The size of ZnO NPs depends on the type of precursor, the solvent, the pH, and the temperature of the reacting solution (Padmavathy & Vijayaraghavan 2008). In this study, ZnO NPs were prepared by two methods (mechano-chemical and sol–gel methods). As was reported earlier, the ZnO NPs, synthesized by mechano-chemical methods (ZnO-1), were characterized by XRD and TEM. The diffraction angle and the intensity of the characteristic peaks of the typical ZnO NPs correspond to JCPDS card No. 36-1451. All peak locations and relative peak intensities of the ZnO NPs agree with those of the standard XRD pattern. In addition, there are no peaks of impurities, indicating that they are of high purity. The average crystalline size of the ZnO NPs product, calculated through using Scherrer's equation (Cullity 1978), was found to be 15.8 nm. TEM images were also used to determine the sizes and structural morphologies of the synthesized ZnO NPs. TEM images showed that ZnO NPs are mainly spherical in shape and 10–20 nm in size, which makes this result consistent with XRD data.

The ZnO NPs (ZnO-2) from the sol–gel method were estimated by XRD data (Figure 1). According to the results, the XRD pattern of the typical ZnO NPs also matched JCPDS card No. 36-1451. In addition, there were no diffraction peaks from other types detected, which illustrates high purity. The average size of the ZnO NPs, as estimated through Scherrer's equation, was found to be about 5–7 nm. The SEM micrograph of the ZnO-2 NPs is shown in Figure 2. The morphological observation also demonstrated the nanoparticles'

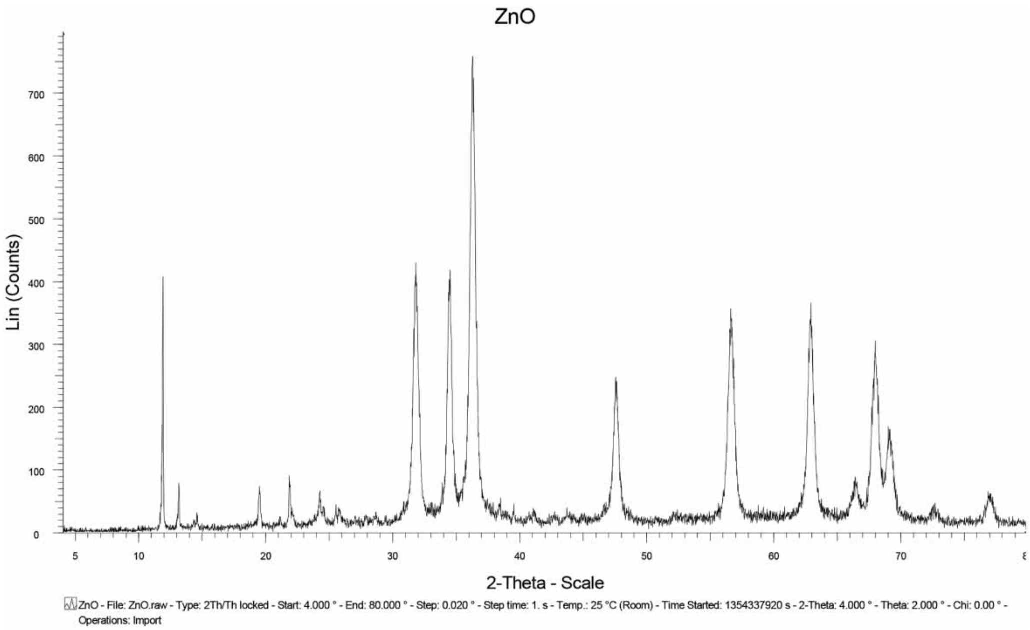

Figure 1 | XRD pattern of ZnO NP synthesized via the sol–gel method.

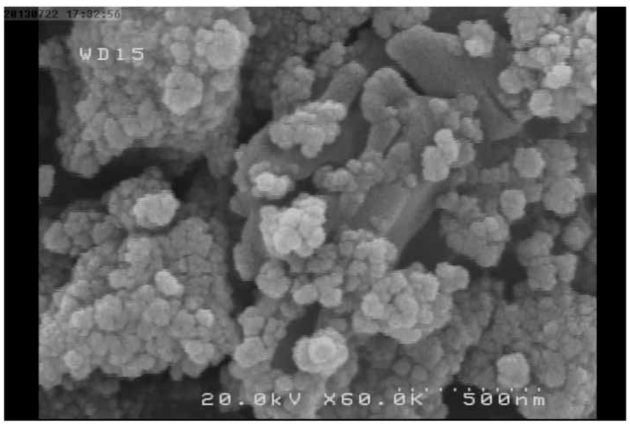

Figure 2 | SEM micrograph of ZnO NP synthesized via the sol–gel method.

Table 2 | Cultured bacteria from municipal wastewaters from two stations

	Bacteria	
Wastewater	**Before disinfection**	**After disinfection**
Station I (chlorine disinfection)	*Escherichia coli*	*Klebsiella pneumoniae*
	Proteus	
	Klebsiella pneumoniae	
Station II (UV disinfection)	*Klebsiella pneumoniae*	*Staphylococcus epidermidis*
	Escherichia coli	
	Staphylococcus epidermidis	

hexagonal wurtzite structure. Furthermore, the image shows the agglomeration of particles, which was much higher in this method of preparation than in the mechano-chemical method.

Separation of bacteria from wastewater

The municipal wastewater samples were collected from two different wastewater stations before and after the disinfection process. As mentioned above, chlorine is applied in station I and UV is used in station II for disinfection. The samples were directly transferred to the laboratory at 4 °C. Then, the samples were cultured evenly on EMB and blood agar medium. As Table 2 shows, before disinfection, Gram-negative bacteria (*K. pneumoniae*, *E. coli,* and *Proteus*) were found in station I samples, and *K. pneumoniae*, *E. coli* and Gram-positive bacteria (*S. epidermidis*) were separated from station II samples. After the chlorine disinfection process, *K. pneumoniae* was the only bacterium that remained, and after UV disinfection *S. epidermidis* was the only bacterium detected.

Antibacterial activity of ZnO NPs

In this study, the antibacterial activity of various concentrations (0.01–50 mM) of ZnO NPs (ZnO-1 and ZnO-2) towards the wastewater separated bacteria was investigated by such bacteriological tests as disk diffusion assay, MIC, and MBC. Disk diffusion assay of ZnO NPs against *K. pneumoniae*, *E. coli*, and *Proteus* (separated from station I) is shown on the basis of the inhibition zone (mm) size in Tables 3(a) and 3(b). Antibacterial activity of ZnO-1 NPs produces zones of inhibition of 8, 10, and 12 mm on *E. coli* at 15, 25, and 50 mM concentrations and 10 and 11 mm on *Proteus* at 25 and 50 mM, respectively. For the ZnO-2 NPs, it produces a zone of inhibition of 10 mm on both *E. coli* and *Proteus* at 50 mM concentration. In the case of *K. pneumoniae*, both ZnO NPs do not show any activity.

As shown in Tables 4(a) and 4(b), antibacterial activity of both ZnO NPs can provide maximum zone of inhibition against *E. coli* at 50 mM concentration. Antibacterial activity of ZnO NPs produces the best zone of inhibition against *S. epidermidis* (18 mm) and (21 mm) at 50 mM concentration of ZnO-1 and ZnO-2, respectively. No inhibitory effect was found against *K. pneumoniae* at concentrations less than 50 mM, whereas, at 50 mM concentration of ZnO-2 NPs, the zone of inhibition produces 9 mm on *K. pneumoniae*. According to the results, ZnO NPs give the best activity against Gram-positive bacteria, *S. epidermidis*.

In addition, the MIC and MBC of ZnO NPs for all bacteria are summarized in Table 5. As Table 5 shows, antibacterial activity of ZnO NPs against *E. coli* was highest among Gram-negative bacteria. The MIC and MBC for both ZnO NPs was calculated to be 203 μg mL^{-1} and >1,017 μg mL^{-1} for *S. epidermidis*, respectively.

Table 3 | Zone of inhibition (mm) against *K. pneumoniae*, *E. coli*, and *Proteus* (separated from station I) by ZnO-1 and ZnO-2 NPs

	Zone of inhibition (mm)		
Concentration of ZnO-1 (mM)	***K. pneumoniae***	***E. coli***	***Proteus***
ZnO-1			
0.01	–	–	–
1	–	–	–
2.5	–	–	–
5	–	–	–
10	–	–	–
15	–	8	–
25	–	10	10
50	–	12	11

	Zone of inhibition (mm)		
Concentration of ZnO-2 (mM)	***K. pneumoniae***	***E. coli***	***Proteus***
ZnO-2 NPs			
0.01	–	–	–
1	–	–	–
2.5	–	–	–
5	–	–	–
10	–	–	–
15	–	–	–
25	–	–	–
50	–	10	10

(–) No activity in these concentrations.

Table 4 | Zone of inhibition (mm) against *K. pneumoniae*, *E. coli*, and *S. epidermidis* (separated from station II) by ZnO-1 and ZnO-2 NPs

	Zone of inhibition (mm)		
Concentration of ZnO-1(mM)	***K. pneumoniae***	***E. coli***	***S. epidermidis***
ZnO-1			
0.01	–	–	–
1	–	–	–
2.5	–	–	10
5	–	–	12
10	–	10	12
15	–	10	15
25	–	12	16
50	–	14	18

	Zone of inhibition (mm)		
Concentration of ZnO-2 (mM)	***K. pneumoniae***	***E. coli***	***S. epidermidis***
ZnO-2 NPs			
0.001	–	–	–
1	–	–	–
2.5	–	–	–
5	–	–	8
10	–	–	12
15	–	10	15
25	–	12	18
50	9	13	21

(–) No activity in these concentrations.

DISCUSSION

The lack of water and the environmental quality of water are regarded as two important problems for the near future. Advances in the field of nanotechnology may show new ways for treatment of water and wastewater. This report evaluated

Table 5 | Determination of MIC and MBC of ZnO-1 and ZnO-2 NPs against *K. pneumoniae*, *E. coli*, *S. epidermidis,* and *Proteus* bacteria

	Station I		Station II	
Bacteria	**MIC (μg mL^{-1})**	**MBC (μg mL^{-1})**	**MIC (μg mL^{-1})**	**MBC (μg mL^{-1})**
ZnO-1				
K. pneumoniae	–	–	–	–
E. coli	1,017	> 1,017	2,035	> 2,035
S. epidermidis	■	■	203	>1,017
Proteus	–	–	■	■
	Station I		**Station II**	
Bacteria	**MIC (μg mL^{-1})**	**MBC (μg mL^{-1})**	**MIC (μg mL^{-1})**	**MBC (μg mL^{-1})**
ZnO-2 NPs				
K. pneumoniae	–	–	–	–
E. coli	1,017	> 1,017	1,017	> 2,035
S. epidermidis	■	■	203	1,017
Proteus	–	–	■	■

The black squares show the bacterium was not present in that station.
(–) No activity in these concentrations.

the relative antibacterial activity of ZnO NPs with average sizes of 15.8 nm and 5–7 nm, which were synthesized by mechano-chemical and sol–gel, respectively, on bacteria separated from wastewater. In our study, the size of the synthesized ZnO NPs from these two methods was compatible with those of others (Spanhel & Anderson 1991; Meulenkamp 1998; Ao *et al.* 2006; Shen *et al.* 2006; Lu *et al.* 2008). Also, in our study, the agglomerations of sol–gel were much higher than the mechano-chemical method. The aggregation observed in our study was also cited in other reports for ZnO and, therefore, is considered to be an innate property of uncoated NPs (Adams *et al.* 2006a; Keller *et al.* 2010; Tso *et al.* 2010).

According to the results, both ZnO NPs proved to have MBC and MIC activities against *E. coli* and *S. epidermidis* but they had no effect on *K. pneumoniae* and *Proteus* bacteria. Only disk diffusion assay showed a moderate effect on *K. pneumoniae* in higher concentration. This result is consistent with the results obtained by Jones *et al.* (2008), Padmavathy & Vijayaraghavan (2008), Gordon *et al.* (2011), Meruvu *et al.* (2011), Wang *et al.* (2012) and Yousef & Danial (2012), but it does not empirically support the results of studies in which ZnO NP has moderate MBC and MIC effects (Yousef & Danial 2012). These authors proposed that the antibacterial activity of ZnO NPs can be influenced by the preparation method in addition to the effect of particle size and the type of bacterial strains. Also, these findings are consistent with the results that demonstrate that ZnO NPs were more toxic toward Gram-positive bacteria (Sawai 2003; Tam *et al.* 2008; Premanathan *et al.* 2011). However, they are contradictory to the ones that used PEGylated ZnO NP, which had less effect on Gram-positive bacteria (Nair *et al.* 2009).

Furthermore, the results showed that the antibacterial activity increased as the concentration of ZnO NPs increased. It seems that ZnO NPs could damage the membrane, lead to the leakage of cytosolic components and destroy the bacterial cells (Wang *et al.* 2012). The results of our study also demonstrated that ZnO NPs exhibited no antibacterial activity in a concentration lower than 1 mM. Sawai maintains that this may be due to lower concentrations of Zn^{2+} ions, which might act as a nutrient (Sawai 2003).

Many researchers have reported the effect of size on the toxicity (Nair *et al.* 2009; Napierska *et al.* 2009). Our result does not show an obvious relationship between toxicity and size of ZnO NPs. Smaller ZnO NPs (from the sol–gel method) were a little bit more effective. It seems that our results may be related to formed aggregations and, thus, the aggregated size could also be regarded as a factor affecting the particles' toxicity (Hsiao & Huang 2011).

The antibacterial mechanism of ZnO NPs is still under investigation. However, several mechanisms have been proposed to interpret the disinfection activity of ZnO NPs (Zhang *et al.* 2007, 2008, 2010). Considering the small size of ZnO NPs, the antibacterial activity might relate to the ability to adhere to the cell wall easily, causing the destruction of its bacterial membrane and leading to the death of the cell (Zhang *et al.* 2013). In addition, the production of different amounts of H_2O_2 and radical oxygen species (ROS) is another proposed mechanism of NPs' activity (Sawai 2003; Zhang *et al.* 2010, 2012, 2013; Raghupathi *et al.* 2011). Gram-positive bacteria were found to be more susceptible to ZnO NPs than were Gram-negative bacteria, probably because the Gram-positive bacteria contain a weaker antioxidant cellular content, making them less resistant to ROS. However, it has been suggested that Zn^{2+} ion, released from dissolution of ZnO NPs, can bind to the bacterial membrane and prolong the lag phase of the growth cycle of bacteria (Atmaca *et al.* 1998).

CONCLUSION

Our experiment sought to explore the antibacterial properties of both ZnO NPs towards important waterborne pathogens, *E. coli*, *Proteus*, *S. epidermidis* and *K. pneumoniae*. The results indicated that the inhibitory effects increased as the concentrations of ZnO NPs increased. Our study does not, however, show the important relationship between the size of ZnO NPs and antibacterial properties. These results show that ZnO NPs can be potentially regarded as an effective antibacterial agent for waterborne pathogens and can be considered as a complementary method for water and wastewater treatment.

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