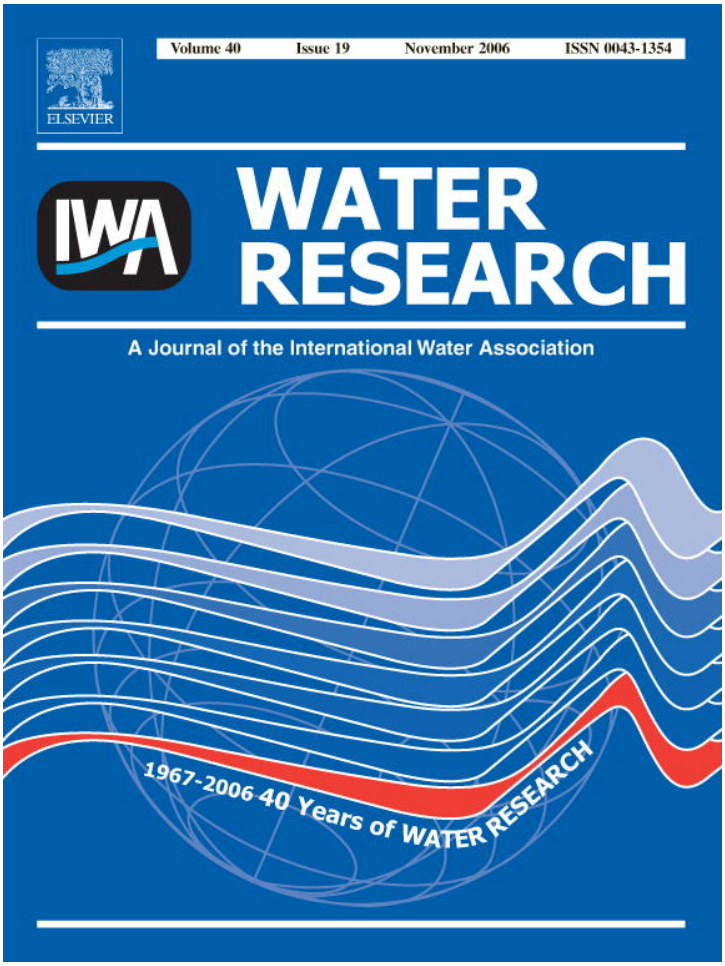

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Comparative eco-toxicity of nanoscale TiO_2, SiO_2, and ZnO water suspensions

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ABSTRACT

The potential eco-toxicity of nanosized titanium dioxide (TiO_2), silicon dioxide (SiO_2), and zinc oxide (ZnO) water suspensions was investigated using Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli* as test organisms. These three photosensitive nanomaterials were harmful to varying degrees, with antibacterial activity increasing with particle concentration. Antibacterial activity generally increased from SiO_2 to TiO_2 to ZnO, and *B. subtilis* was most susceptible to their effects. Advertised nanoparticle size did not correspond to true particle size. Apparently, aggregation produced similarly sized particles that had similar antibacterial activity at a given concentration. The presence of light was a significant factor under most conditions tested, presumably due to its role in promoting generation of reactive oxygen species (ROS). However, bacterial growth inhibition was also observed under dark conditions, indicating that undetermined mechanisms additional to photocatalytic ROS production were responsible for toxicity. These results highlight the need for caution during the use and disposal of such manufactured nanomaterials to prevent unintended environmental impacts, as well as the importance of further research on the mechanisms and factors that increase toxicity to enhance risk management.

1. Introduction

Titanium dioxide (TiO_2), silicon dioxide (SiO_2), and zinc oxide (ZnO) are common additives with a variety of applications. TiO_2 is a good opacifier and is used as a pigment in paints, paper, inks, and plastics. Crystalline SiO_2 is employed in electronics manufacturing as both semiconductor and electrical insulator. The ceramic nature of ZnO permits its function as both pigment and semiconductor. Nanoscale TiO_2, SiO_2, and ZnO offer greater surface area than their bulk counterparts, allowing for improved performance in established applications.

Accompanying the well-established use of TiO_2, SiO_2, and ZnO, research has been conducted on their potential toxicity (Rincon and Pulgarin, 2004; Lonnen et al., 2005). A wealth of information exists on the toxicity of TiO_2 towards bacteria (e.g. Wei et al., 1994; Block et al., 1997; Kwak et al., 2001). TiO_2 is reputed to be toxic to both Gram-negative and Gram-positive bacteria. In a mixed culture experiment, an unidentified Gram-positive *Bacillus subtilis* was less sensitive than a pure culture of Gram-negative *Escherichia coli* to the effects of TiO_2, possibly due to the ability of *B. subtilis* to form spores (Rincon and Pulgarin, 2005). However, other studies have found Gram-positive bacteria to be more sensitive than Gram-negative bacteria to the antibacterial effects of TiO_2 (Fu et al., 2005). The antibacterial properties of TiO_2 have been exploited in water treatment reactors. A concentration of TiO_2 ranging from 100 to 1000 ppm has been reported to completely disinfect water containing 10^5–10^6 *E. coli* cells per ml in 30 min under illuminated conditions (Wei et al., 1994; Maness et al., 1999).

Fewer studies have been initiated on the antibacterial activities of either SiO_2 or ZnO. Bulk SiO_2 has been used as a

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control particle in several studies due to its postulated lack of toxicity towards bacteria (e.g. Liang et al., 2004). ZnO has been reported to exhibit antibacterial activity with Gram-positive *B. subtilis* being more sensitive to its effects than the Gram-negative *E. coli* (Sawai et al., 1995). The minimal inhibitory concentrations ranged from 2000 to 12,500 ppm for *B. subtilis* and 50,000 to 100,000 ppm for *E. coli* depending on particle size (Sawai et al., 1996). While these data suggest that ZnO is much less toxic to *E. coli* than TiO_2, it is not possible to directly compare these studies due to differences in experimental design (e.g., particle size, concentration of bacteria, application of light).

The differential toxicity of TiO_2, SiO_2, and ZnO may be related to the mechanisms by which the particles act on cells. It is documented that these three compounds are photosensitive and produce reactive oxygen species (ROS) in the presence of light (Yeber et al., 2000; Fubini and Hubbard, 2003; Kubo et al., 2005). However, a positive correlation between photocatalytic ROS production and antibacterial activity has been determined only for TiO_2. Light in these reactions is usually provided by specific wavelength high-intensity lamps; however, one study showed that TiO_2 exhibited antibacterial properties when sunlight was the source of illumination (Wei et al., 1994).

In previous studies, TiO_2 particles that were toxic to bacteria ranged in size from tens of nanometers to hundreds of micrometers. It is not currently clear whether particle size is a key determinant of toxicity or whether surface chemistry and morphology are more important. With the rapid emergence of nanoparticles, it is important to identify the factors that accentuate toxicity. Currently, legislation of nanomaterials is limited, mainly due to the lack of toxicological information and the novelty of the field (Hogue, 2005). However, it is crucial that we understand the fate and impact of potential "contaminants" to permit the development of appropriate disposal mechanisms that mitigate the contamination of surface and groundwater resources.

Little published research has focused on the antibacterial effects related to disposal or accidental spillage of TiO_2, SiO_2, and ZnO. Many studies using nanoscale TiO_2 have incorporated solubilising agents (e.g., hydroxyl groups) into the suspension (Kwak et al., 2001) or have immobilised the TiO_2 onto glass (Rincon and Pulgarin, 2004), stainless steel (Yu et al., 2003) or acetate sheets (Lonnen et al., 2005) or have utilized artificial (relatively intense) light sources. While these studies focused on parameters of their particular application, they might not be representative of the effect of raw nanoscale TiO_2 release into the aqueous environment. Therefore, we used nanoparticle water suspensions and natural sunlight to better model natural nanoparticle exposure.

This paper compares and contrasts the toxic effects associated with TiO_2, SiO_2, and ZnO water suspensions using two model bacterial species, Gram-negative *E. coli* and Gram-positive *B. subtilis*. The objectives of this study were to (a) determine the concentrations at which the three suspensions are toxic to our test organisms, (b) determine whether the size of the released nanoparticle affects antibacterial activity, and (c) determine whether natural light stimulates toxicity of the nanoparticles to bacteria.

2. Methods

2.1. Organism cultivation

E. coli DH5α and *B. subtilis* CB310 (courtesy of Dr. Charles Stewart, Rice University, Houston, TX) were maintained on Luria–Bertani (LB) plates. For all experiments, the bacteria were cultivated in a minimal Davis medium (MD). MD is a variation of Davis medium in which the potassium phosphate concentration was reduced by 90% (Atlas, 1993). This medium consisted of 0.7 g K_2HPO_4, 0.2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g Na-citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, and 1 g glucose in 1 l of Milli-Q® at pH 7.0. MD medium was chosen as the antibacterial test medium as previous research has shown that other nano-sized aggregates precipitate out of suspension in media containing high phosphate concentrations (Lyon et al., 2005).

2.2. Preparation of nanoparticle suspensions

TiO_2 (66 nm, 950 nm, and 44 μm advertised particle size), SiO_2 (14 nm, 930 nm, and 60 μm advertised particle size), and ZnO (67 and 820 nm advertised particle size) powders were obtained from Sigma-Aldrich (St. Louis, MO, USA). ZnO powder at 44 μm particle size was obtained from Alfa Aesar (Ward Hill, MA, USA). The 66 and 950 nm TiO_2 are mixtures of anatase and rutile and the 44 μm TiO_2 is almost pure anatase. The advertised particle size was compared to the measured particle size in suspension. Each of the powders was added to 100 ml of Milli-Q® water to obtain a final concentration of 10 g/l and shaken vigorously. The actual size of the particles in suspension in water and in MD was determined using a dynamic light scattering device (Brookhaven Instrument Corporation, Holtsville, NY, USA) for particles below 1 μm diameter, and optical microscopy (Nikon Optiphot, Japan) for those above this limit. All sizes were confirmed using TEM. To facilitate comparative discussion, the three differently sized suspensions obtained for each compound will be termed small, medium, and large, respectively after the relative advertised sizes of the starting materials.

2.3. Assessment of toxicity to bacteria

Petri plates containing liquid MD media were supplemented with appropriate concentrations (10–5000 ppm) of nanoparticle suspensions to achieve a final volume of 5 ml prior to inoculation with an overnight culture of *B. subtilis* or *E. coli* ($OD_{600} = 0.002$). Antibacterial activity assays were conducted in the presence of sunlight with the small-sized particle suspensions. To obtain data on the effect of size and light on toxicity, suspensions were added at pre-determined toxic concentrations. Control plates were prepared containing only MD medium and bacteria. Plates were sealed with Parafilm (American National Can, Chicago, IL, USA) and wrapped in aluminium foil to simulate dark conditions where required. All plates were placed on a rocker platform (Bell Company Biotechnology, Vineland, NJ, USA) to maintain the nanoparticles in suspension and left in direct sunlight for 6 h (9 AM to 3 PM). The experiments were conducted in the window of a southeast facing laboratory on bright days (23 °C average

temperature, UV Index 6–7) in October in Houston, TX (29°N, 95°W). The average outdoor incident luminescence during the test periods was 50.4 klux/h, with the indoor values being similar, as the windows had no special coating. Cultures were diluted to achieve cell concentrations of approximately 10^3 CFU/ml, spread onto LB plates, and left to grow at 36 °C for 14–20 h. Colonies were counted and compared to control plates to calculate percentage growth inhibition. All treatments were prepared in duplicate and repeated on three separate occasions.

3. Results and discussion

3.1. *Characterization of suspensions*

The true size of the particles in suspension was significantly different than the advertised size of the starting powders (Table 1). This phenomenon has been reported by others (Hristovski et al., 2005). Our suspensions in water and MD appeared to contain similarly sized particles regardless of the advertised size of the starting material. Overall, the small suspensions contained particles that were one order of magnitude larger than the advertised size. Conversely, the medium and large suspensions contained particles smaller than the advertised size. The sizes of the particles were similar in water and in MD. The discrepancies in size are mainly due to aggregation of the particles and a certain amount of uncertainty in the manufacturing process.

3.2. *Determination of antibacterial concentrations*

Although antibacterial activity increased with dose for all treatments (Table 2), the two bacterial species behaved differently upon exposure to the same levels of nanoparticle suspensions.

Increasing TiO_2 concentrations showed a gradual increase in toxicity towards *E. coli* with 72% growth reduction in cells exposed to 5000 ppm (Table 2). In contrast, *B. subtilis* were more susceptible with 1000 ppm TiO_2 resulting in 75% growth reduction and 2000 ppm resulting in 99% growth reduction. The concentrations of TiO_2 required to kill bacteria were greater than in previously published studies (Wei et al., 1994; Rincon and Pulgarin, 2005). The difference in toxicity thresholds may be related to particle size or to the light source employed during cell growth. Previous studies used high-intensity lamps emitting light between 300 and 400 nm that potentially generate more ROS (Goswami et al., 1997). With the application of very high light intensities, TiO_2 antibacterial activity has been elicited at concentrations as low as 0.001 ppm for Degussa P-25 particles with an advertised size of 21 nm (Matsunaga and Okochi, 1995). The actual size of those particles in suspension was not reported. This study suggests that light intensity modulates the toxicity of TiO_2.

SiO_2 was the least toxic of the nanomaterials tested and relatively high concentrations were required to achieve a reduction in cell growth. Addition of SiO_2 at 5000 ppm resulted in 99% growth reduction of *B. subtilis* (Table 2). This indicates that nanosized SiO_2 is not as inert in bacterial systems as implied in other studies working with microsized bulk SiO_2 (Liang et al., 2004). Interestingly, *E. coli* was less susceptible to the effects of SiO_2 with 5000 ppm achieving only 48% growth reduction.

At 10 ppm, ZnO resulted in 90% growth reduction of *B. subtilis* but only 48% growth reduction in *E. coli* resulted at 1000 ppm ZnO. The antibacterial concentrations of ZnO reported here are considerably lower than in other published studies (two orders of magnitude lower for *B. subtilis* and one order of magnitude for *E. coli*). These differences may be attributable to the smaller sized particles or the relatively low-salt/protein growth medium utilized in our studies (which minimizes the potential for nanoparticle coagulation and precipitation).

Overall, these data showed that the Gram-positive *B. subtilis* was more sensitive to the addition of all nanoparticles than Gram-negative *E. coli*. While this is in agreement with previously published reports on the antibacterial properties of ZnO (Sawai et al., 1995), it is in contrast with some published reports on the antibacterial properties of TiO_2 (Rincon and Pulgarin, 2005). *B. subtilis* is generally considered

Table 1 – Measurement of particle size ranges and mean size for all suspensions

Suspension	Terminology	Advertised particle size (nm)	Actual particle size range in suspension (nm)	Actual mean particle size in suspension (nm)
TiO_2	Small	66	175–810	330
	Medium	950	240–460	320
	Large	44,000	1000	1000
SiO_2	Small	14	135–510	205
	Medium	930	380–605	480
	Large	60,000	10,000–75,000	47,000
ZnO	Small	67	420–640	480
	Medium	820	570–810	780
	Large	44,000	1000–13,000	4000

Small and medium suspensions were measured by DLS and large by optical microscopy.

Table 2 – Percentage growth inhibition when (advertised) small particle suspensions were applied to *B. subtilis* and *E. coli* in light at various concentrations (n.d. = not determined)

Treatment		Percentage growth inhibition at specified concentration (±1 standard deviation, $n = 6$)						
		10 ppm	50 ppm	100 ppm	500 ppm	1000 ppm	2000 ppm	5000 ppm
B. subtilis	TiO_2 (330 nm)	n.d.	0	0	0	75±6.6	99±0.9	n.d.
	SiO_2 (205 nm)	n.d.	0	0	0	7±4.7	84±9.9	99±1.8
	ZnO (480 nm)	90±4.4	98±0.8	98±1.4	98±0.8	n.d.	n.d.	n.d.
E. coli	TiO_2 (330 nm)	n.d.	0	0	15±4.2	44±7.0	46±11.3	72±9.4
	SiO_2 (205 nm)	n.d.	0	0	15±6.4	19±8.3	32±10.1	48±8.5
	ZnO (480 nm)	14±3.5	22±6.5	28±4.9	38±8.9	48±7.7	n.d.	n.d.

Mean particle size for each nanoparticle is added in parentheses.

to be less sensitive to the effects of TiO_2 due to its ability to form spores and its cell wall structure. More research is required to determine why *B. subtilis* was more sensitive than *E. coli* to nanoparticle suspensions in this and other studies (Sawai et al., 1995).

3.3. *Effect of particle size on antibacterial activity*

Advertised particle size did not affect antibacterial activity, since all powders resulted in similarly sized particles in suspension, regardless of the advertised powder size. At any given concentration, a compound was either bactericidal or not toxic for all three advertised sizes tested (Fig. 1). Previous studies of the effect of nanoparticle size on cytotoxicity have reported variable results, from a lack of significant effect (Yamamoto et al., 2003) to increasing toxicity with decreasing particle size (Sawai et al., 1996). Theoretical considerations suggest that smaller particles with higher specific surface area should be more toxic, but comparison between published studies may be confounded by differences in external factors, including light intensity, surface chemistry, particle morphology and bacterial concentration. In this work, the advertised size of nanoparticles used to prepare the suspensions did not significantly affect toxicity (Fig. 1) despite advertised sizes ranging over 3–4 orders of magnitude (Table 1). However, it should be noted that the mean actual particle sizes in suspension were generally similar, varying only within one order of magnitude (Table 1). The similar true size of particles in suspension precludes us from evaluating toxicity as a function of true size. As expected, the similar sizes of particles in suspension resulted in similar antibacterial activities. These data do highlight that advertised particle size may be a poor indicator of true particle size in suspension and consequently, also of potential toxicity.

3.4. *Effect of light on antibacterial activity*

Overall, illumination seemed to enhance the antibacterial activity of TiO_2 but not ZnO or SiO_2 (Fig. 2). For ZnO, there was near-complete inhibition of *B. subtilis* growth (even at the lowest tested dose of 10 ppm) under both dark and illuminated conditions (Fig. 2A). In contrast, *E. coli* was less susceptible to ZnO exposure, with minimal growth inhibition

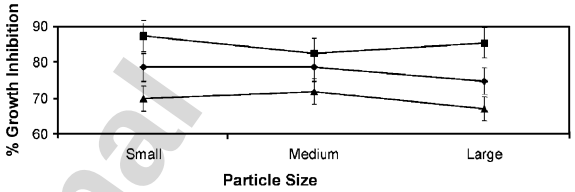

Fig. 1 – The increase in nanoparticle advertised size (Table 1) did not affect the antibacterial activity of the suspensions (Symbols: ■, ZnO; ◆, TiO_2; and ▲, SiO_2). Error bars showing that values deviated from the mean by a maximum of 5%.

under dark conditions (Fig. 2B). The difference in response between these two species is unclear and may reflect differences in cell physiology, metabolism, or degree of contact. The absence of this sensitive response by *B. subtilis* to their nanoparticles (see below) suggests that the mechanism(s) of toxicity might also differ depending on the type of nanoparticle.

The antibacterial activity of TiO_2 towards both bacterial species was significantly greater ($p < 0.05$) in the presence of light than in the dark, and this difference was more pronounced for *B. subtilis*. Specifically, the degree of inhibition for *B. subtilis* was 2.5-fold greater in the presence than in the absence of light (Fig. 2A), compared to 1.8-fold for *E. coli* (Fig. 2B). The greater inhibition in the presence of light supports the notion that the antibacterial activity of TiO_2 was related to photocatalytic ROS production (Maness et al., 1999). While cell death with TiO_2 was less pronounced in the dark, it still occurred, indicating that an additional mechanism is involved. Similar results have been reported from mammalian cytotoxicity studies, where TiO_2 exerted oxidative stress in the dark under non-photocatalytic conditions (Gurr et al., 2005).

Similar to results observed with TiO_2, SiO_2 was toxic to both *E. coli* and *B. subtilis* under both light and dark conditions, and cell growth inhibition appeared higher in the presence of light. However, when analyzed statistically at 95% confidence level, cell growth inhibition with SiO_2 was similar under both dark and light conditions, indicating that light had an insignificant effect in increasing the toxicity of SiO_2 (Fig. 2A and B).

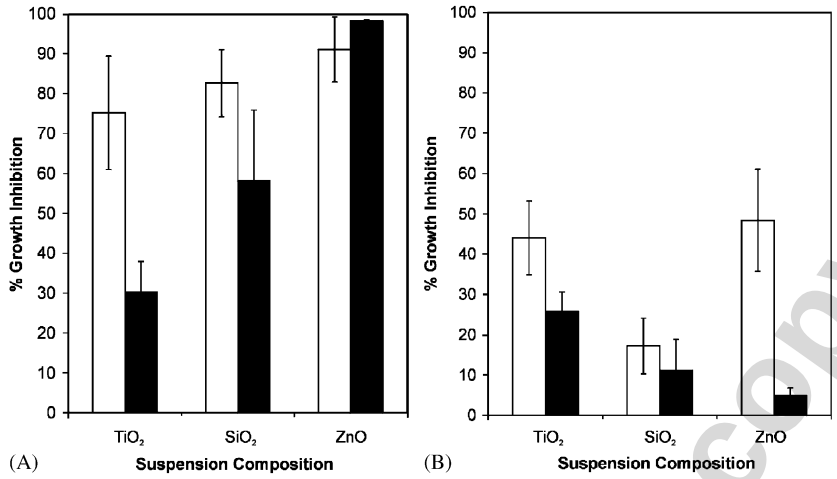

Fig. 2 – The effect of illumination on antibacterial activity of (advertised) small particle suspensions (Table 1) towards (A) *B. subtilis* and (B) *E. coli* (Symbols: ⩽, light, ■, dark). Error bars represent ± 1 standard deviation from the mean, $n = 6$.

Since light is needed to produce photocatalytic ROS, toxicity to organisms exposed under dark conditions must be attributed to an as yet undetermined mechanism(s). This underscores the need for further research on nanomaterial–cell interactions and cytotoxicity mechanisms that prevail in the dark. Potential mechanisms that should be investigated include oxidative stress via ROS formation, organic radicals generated in the absence of light, and the role of nanomaterials in disruption of membrane integrity.

This study examined the behavior of pure cultures of organisms in a medium optimized for bacterial growth. This may not give an accurate reflection of the toxicities of TiO_2, SiO_2, and ZnO water suspensions that would occur in natural systems with higher ionic strength that might promote removal of the nanomaterial suspensions by coagulation and precipitation.

4. Conclusions

Nanosized TiO_2, SiO_2, and ZnO water suspensions exhibited antibacterial properties towards *B. subtilis* and to a lesser extent to *E. coli*. Overall, antibacterial effects increased from SiO_2 to TiO_2 to ZnO. The toxicity displayed by nanosized SiO_2 towards *B. subtilis* should be noted, given previous studies indicating that microsized bulk SiO_2 was inert.

Even though the ranges of differently sized powders were used (10^1–10^4 nm), the consequence of particle size could not be effectively measured in this study. The aggregation of particles in water led to their true size in suspension differing widely from that of the dry powders. The resulting suspended particles were all similarly sized and exhibited similar antibacterial activity. This precluded discerning the effect of size on toxicity.

Before definitive conclusions can be drawn regarding the effect of light on toxicity, further studies should be performed. Although, all the nanoparticles tested are capable of producing toxic ROS in the presence of light, the inhibitory effects observed under dark conditions suggest that additional, as yet undetermined mechanisms might contribute to toxicity. The results of this study highlight the need for safe disposal protocols for each of these compounds. Their release into surface or ground waters could have detrimental effects to ecosystem health.

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