Comparative toxicity of nano-scale TiO_2, SiO_2 and ZnO water suspensions

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Abstract TiO_2, SiO_2 and ZnO are common additives with improved applications at the nanoscale. The antibacterial activity of TiO_2, which has important ecosystem health implications, is well understood. However, less attention has been paid to the antibacterial activity of SiO_2 and ZnO despite them also producing reactive oxygen species. This paper explores the relative toxicity of TiO_2, SiO_2 and ZnO water suspensions towards bacteria (*B. subtilis*, *E. coli*) and the eukaryotic *Daphnia magna*. These three photosensitive nanomaterials were hazardous to all test organisms, with toxicity increasing with particle concentration. Toxicity of the three compounds decreased from ZnO to TiO_2 to SiO_2 and *Daphnia* were most susceptible to their effects. Nominal particle size did not affect the toxicity of these compounds. Antibacterial activity was noted under both dark and light conditions indicating that mechanisms additional to ROS production were responsible for growth inhibition. 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.

Keywords Antibacterial; *Bacillus subtilis*; *Daphnia magna*; *Escherichia coli*; nanometer; size

Water Science & Technology **Vol 54 No 11–12 pp 327–334** © IWA Publishing 2006

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 a semiconductor and an electrical insulator. The ceramic nature of ZnO permits its function as both a pigment and a semiconductor. It also has the ability to absorb ultraviolet light resulting in its incorporation into sunscreens. 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 TiO_2 bacterial toxicity (Wei *et al.*, 1994; Block *et al.*, 1997; Kwak *et al.*, 2001). The effect of initial bacterial concentration, pH, inorganic ion concentration, humidity and oxygen on the antibacterial activity of TiO_2 have all been investigated (Wei *et al.*, 1994; Goswami *et al.*, 1997; Rincon and Pulgarin, 2004). TiO_2 is reputed to be toxic to both Gram-negative and Gram-positive bacteria, although Gram-positive bacteria are less sensitive due to their ability to form spores (Wei *et al.*, 1994). A positive spin has been given to the antibacterial properties of this compound culminating in its use in water treatment reactors. The concentration of TiO_2 required to exhibit strong antibacterial effects lies between 100 and 1000 ppm (Wei *et al.*, 1994; Matsunaga and Okochi, 1995; Maness *et al.*, 1999; Rincon and Pulgarin, 2005).

Fewer studies have been initiated on the antibacterial activities of either SiO_2 or ZnO. SiO_2 has been used as a control particle in several studies due to its postulated lack

doi: 10.2166/wst.2006.891

of toxicity towards bacteria (Liang *et al.*, 2004). Zinc oxide is considered to be less toxic than TiO_2 with minimal inhibitory concentrations reported to range of 2000–12,500 ppm for *B. subtilis* and 50,000–100,000 ppm for *E. coli* depending on particle size (Sawai *et al.*, 1995). Conversely to TiO_2, ZnO is considered most toxic towards Gram-positive bacteria.

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 produce reactive oxygen species (ROS; Yeber *et al.*, 2000; Fubini and Hubbard, 2003; Kubo *et al.*, 2005). However, the positive correlation between ROS production and antibacterial activity has been determined only for TiO_2.

In previous studies, the TiO_2 particles found to be toxic to bacteria have ranged in size from tens of nanometres to hundreds of micrometres. It is not currently clear whether particle size is a key criterion in determining a particle's toxicity or whether surface chemistry and morphology are more important. With the rapid emergence of nanoparticles, it is imperative for this to be clarified. Currently, legislation of nanomaterials is limited, mainly due to the lack of information and the novelty of the field (Hogue, 2005). However, it is crucial that we understand the fate of potential toxins to allow correct disposal mechanisms to be developed and to prevent the contamination of surface and groundwater resources.

Little 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 solublising agents, e.g. hydroxyl groups, into the suspension (Kwak *et al.*, 2001) or have immobilised the TiO_2 on glass (Rincon and Pulgarin, 2004), stainless steel (Yu *et al.*, 2003) or acetate sheets (Lonnen *et al.*, 2005). Whilst these studies focus on concerns in their particular application, they may not be a valid assessment of the effect of nanoscale TiO_2 release into the aqueous environment as a raw material.

Through the use of two model bacterial species (Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis*) and the common water quality test eukaryotic organism *Daphnia magna*, we aim to compare and contrast the toxic effects associated with TiO_2, SiO_2 and ZnO water suspensions. The objectives of this paper are: (a) to determine the concentrations at which the three suspensions are toxic to our test organisms; (b) to determine whether the size of the nanoparticles affects antibacterial activity; and (c) to determine whether light stimulates toxicity of the nanoparticles to bacteria.

Methods

Organism cultivation

Escherichia coli DH5α and *Bacillus subtilis* CB310 (courtesy of Dr. Charles Stewart, Rice University, Houston, TX) were maintained on Luria-Bertani (LB) plates. For the experiments, they were cultivated in a modified Davis medium, which was termed Minimal Davis (MD) (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, and 0.1 g $MgSO_4.7H_2O$ in 1 L of Milli-Q® water which was autoclaved and amended with filter-sterilized glucose solution to achieve 1 g/L glucose concentration. *Daphnia magna* were obtained from North Carolina Biological Supplies (North Carolina, USA) and cultivated over a four week period to obtain a thriving population.

Preparation of nanoparticle suspensions

TiO_2 (66 nm, 950 nm and 44 μm particle size), SiO_2 (14 nm, 930 nm, and 60 μm particle size), and ZnO (67 nm, 820 nm, and 44 μm particle size) powders were obtained from Sigma-Aldrich (St. Louis, MO, USA). 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. To permit comparative discussion, the three differently sized suspensions obtained for each compound will be termed small, medium and large, respectively, after the relative size of the starting materials. Particle size range was estimated 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.

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 predetermined 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 six hours. Cultures were diluted to achieve cell concentrations of approximately 10^3 CFU/mL, spread onto LB plates and left to grow at 36 °C overnight. 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.

Assessment of toxicity to *Daphnia magna*

TiO_2 and SiO_2 were tested at 1 ppm, 10 ppm and 20 ppm. ZnO was tested at 0.2 ppm, 0.5 ppm and 1 ppm. Spring water modified with nanoparticle suspensions at the above concentrations were added to 1 L containers and supplemented with one *Daphnia* food pellet prior to the addition of 10 *Daphnia.* Containers were left at room temperature and observed for eight days after which the survivorship was recorded. Each variable was prepared in triplicate.

Results and discussion

Characterization of suspensions

The size of particles in suspension was significantly different than the nominal size of the starting powders (Table 1). This phenomenon has been reported by others (Hristovski *et al.*, 2005). The suspensions appeared to contain similarly sized particles regardless

Table 1 Measurement of particle size ranges and mean size for all suspensions. Small and medium suspensions are measured by DLS and large suspensions are measured by optical microscopy

Suspension	Relative term given to suspension	Published size of particles	Actual range of particle sizes within suspension	Actual mean size of particles within suspension
TiO_2	small	66 nm	175–810 nm	330 nm
	medium	950 nm	240–460 nm	320 nm
	large	44 μm	1 μm	1 μm
SiO_2	small	14 nm	135–510 nm	205 nm
	medium	930 nm	380–605 nm	480 nm
	large	60 μm	10–75 μm	47 μm
ZnO	small	67 nm	420–640 nm	480 nm
	medium	820 nm	570–810 nm	780 nm
	large	44 μm	1–13 μm	4 μm

of the advertised size of the starting material. Overall, the small suspensions contained particles that were an order of magnitude larger than the published size. Conversely, the medium and large suspensions contained particles smaller than the published size. The discrepancies in size are mainly due to aggregation of the particles and a certain amount of uncertainty in the manufacturing process.

Determination of antibacterial concentrations

Although antibacterial activity generally increased with dose for all treatments (Tables 2 and 3), the two bacterial species behaved differently upon exposure to the same levels of nanoparticle suspensions. At 10 ppm, ZnO resulted in 90% growth reduction of *B. subtilis* but only 48% growth reduction in *E. coli* resulted at 100 times this concentration.

SiO_2 was the least toxic of the nanomaterials tested. However, at 5000 ppm it resulted in 99% growth reduction of *B. subtilis*. This indicates that SiO_2 is not inert in bacterial systems as previously implied in other studies (Liang *et al.*, 2004). *E. coli* were less susceptible to the effects of SiO_2, with 5000 ppm achieving only 48% growth reduction.

TiO_2 showed a gradual increase in toxicity towards *E. coli* with 72% growth reduction in cells exposed to 5000 ppm. In contrast, *B. subtilis* were more susceptible and 2000 ppm TiO_2 resulted in 99% growth reduction. More research is required to determine why *B. subtilis* appeared more sensitive than *E. coli* to nanoparticle suspensions in this study.

The concentrations of TiO_2 required to kill bacteria were greater than in certain published studies (Wei *et al.*, 1994; Rincon and Pulgarin, 2005). The difference in toxicity thresholds may be related to particle size or the light source present during cell growth. Many previous studies used higher-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 shown at concentrations as low as 0.001 ppm for Degussa P-25 particles with a nominal size of 21 nm (Matsunaga and Okochi, 1995). The actual size of those particles in suspension was not reported.

Determination of toxic levels towards *Daphnia magna*

As was the case with bacteria, ZnO was the most toxic to *Daphnia* of the three compounds tested. At a concentration of 1 ppm only ZnO was lethal. Further tests showed that ZnO was toxic to *Daphnia* at 0.5 and 0.2 ppm, resulting in 100% and 73% mortality respectively (data not shown). Mortality with 1 ppm SiO_2 and TiO_2 resembled that observed in the controls with no suspension added. At 10 ppm, SiO_2 was significantly toxic with 70% mortality. However, TiO_2 caused only 40% mortality at 20 ppm. It is likely that greater percentage mortality would be achieved with higher TiO_2 concentrations.

These results establish that ZnO water suspensions are extremely toxic and detrimental to the survival of *Daphnia magna*. TiO_2, SiO_2 and ZnO are toxic to *Daphnia magna* at approximately two orders of magnitude lower concentrations than those at which they are toxic to *E. coli* and *B. subtilis*. This could be due to the filter-feeding nature of the

Table 2 Percentage growth inhibition when small suspensions were applied to *B. subtilis* in the light at various concentrations (nd = not determined)

Treatment	Percentage growth inhibition at specified concentration						
	10 ppm	50 ppm	100 ppm	500 ppm	1000 ppm	2000 ppm	5000 ppm
TiO_2	nd	0	0	0	75 ± 6.6	99 ± 0.9	nd
SiO_2	nd	0	0	0	7 ± 4.7	84 ± 9.9	99 ± 1.8
ZnO	90 ± 4.4	98 ± 0.8	98 ± 1.4	98 ± 0.8	nd	nd	nd

Table 3 Percentage growth inhibition when small suspensions were applied to *E. coli* in the light at various concentrations (nd = not determined)

Treatment	Percentage growth inhibition at specified concentration						
	10 ppm	50 ppm	100 ppm	500 ppm	1000 ppm	2000 ppm	5000 ppm
TiO_2	nd	0	0	15 ± 4.2	44 ± 7.0	46 ± 11.3	72 ± 9.4
SiO_2	nd	0	0	15 ± 6.4	19 ± 8.3	32 ± 10.1	48 ± 8.5
ZnO	14 ± 3.5	22 ± 6.5	28 ± 4.9	38 ± 8.9	48 ± 7.7	nd	nd

Daphnia. Daphnia ingest water to feed and therefore ingest the suspensions while bacteria are merely suspended in the water and are unlikely to assimilate particles greater than 5 nm (Kloepfer *et al.*, 2005).

Effect of particle size on antibacterial activity

At any given concentration, a compound was either toxic or not toxic for all three sizes tested. 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 decreasing toxicity to *B. subtilis* with increasing ZnO particle size (Sawai *et al.*, 1996). Whilst theoretical considerations suggest that smaller particles with higher specific surface area should be more toxic, comparison between published studies may be confounded by differences in external factors, including light intensity and amount of bacteria. In this work, the nominal size of nanoparticles used to prepare the suspensions did not significantly affect toxicity (Figure 1) despite nominal size ranging over 3–4 orders of magnitude (Table 1). However, it should be noted that the mean actual particle size in suspension generally varied only within one order of magnitude (Table 1). Apparently, such differences in nanoparticle size were insufficient to significantly affect toxicity.

Effect of light and dark on antibacterial activity

Illumination enhanced the toxicity of the nanoparticle suspensions tested (Figure 2), with the exception of ZnO that near-completely inhibited the growth of *B. subtilis* (even at the lowest dose of 10 ppm) under both dark and illuminated conditions (Figure 2a). Whether light exacerbates ZnO toxicity to *B. subtilis* at levels below 10 ppm was not investigated. When *E. coli* was exposed to ZnO, a gradual increase in growth inhibition with increasing dose was noted in the presence of light. However, exposure to ZnO in the dark resulted in a low but constant growth inhibition (4–10%) that was not dose-dependent (data not shown). We do not have an explanation for this phenomenon.

The antibacterial activity of TiO_2 towards both bacterial species was greater in the presence of light than in the dark, and this difference was more pronounced for *B. subtilis.*

Size were insufficient to significantly affect toxicity.

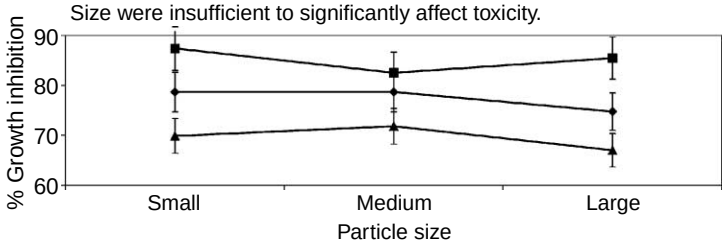

Figure 1 The increase in particle size of the raw materials did not affect the antibacterial activity of the suspensions (symbols: ■ Zn; ◆ TiO_2; ▲ SiO_2). Error bars show that values deviated from the mean by a maximum of 5%

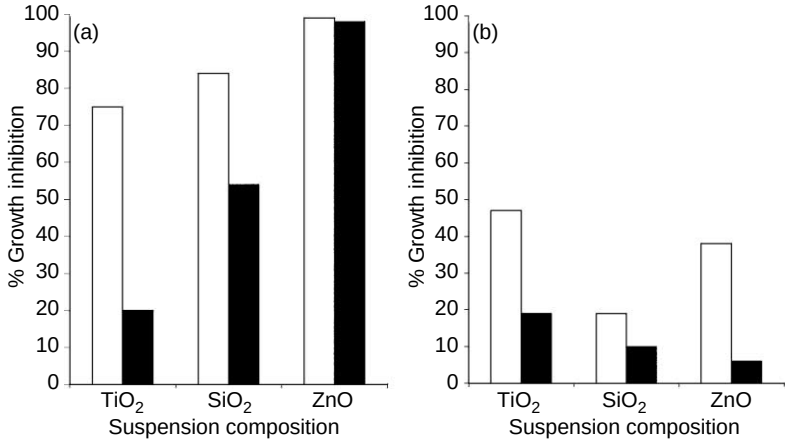

Figure 2 The effect of illumination on antibacterial activity towards: (a) *B. subtilis*; and (b) *E. coli* (symbols: ≤ light; ′ dark). Plotted are growth inhibitions averaged over three repeats of the experiment, all sets showed similar values

Specifically, the degree of inhibition for *B. subtilis* was 3.7 times greater in the presence than in the absence of light (Figure 2a), compared to 2.5 times for *E. coli* (Figure 2b). The greater inhibition noted in the presence of light supports the notion that the antibacterial activity of TiO_2 is related to ROS production, which occurs only occurs in the presence of light (Maness *et al.*, 1999). Yet, cell death with TiO_2 whilst less pronounced did also occur in the dark indicating that an additional mechanism is involved.

Similar to results with TiO_2, SiO_2 was toxic to both *E. coli* and *B. subtilis* under both light and dark conditions, and cell growth inhibition was higher in the presence of light. However, light had a smaller effect in increasing the toxicity of SiO_2. Specifically, the degree of inhibition was 1.6 times greater in the presence than in the absence of light for *B. subtilis* (Figure 2a), and 1.9 times for *E. coli* (Figure 2b). Whereas we cannot rule out the production of small amounts of ROS in dark treatments (due to light intrusion during set up and sampling periods), it is unlikely that sufficient ROS was generated in the dark incubations to solely account for the observed toxicity. Thus, toxicity must be attributed to an as yet undetermined mechanism(s) not involving oxidative stress by ROS. This underscores the need for further research on nanomaterial-cell interactions and cytotoxicity mechanisms that could prevail in the dark.

Please note that this study examined the behaviour 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 in natural systems with different ionic compositions that might promote removal of the nanomaterial suspensions by coagulation and precipitation.

Conclusions

Nanosized TiO_2, SiO_2 and ZnO water suspensions were toxic to *Daphnia magna 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 SiO_2 towards *B. subtilis* should be noted given previous studies indicating that it may be inert. Attention should also be drawn to the toxicity of ZnO towards *Daphnia magma* at 0.2 ppm. Similar effects may be observed with filter feeding organisms higher up in the food chain, e.g. molluscs. Killing such filter feeders would be detrimental to water quality due to their role as 'polishers' that clarify waters in rivers and lakes.

Over the nominal particle size range tested ($10^1 - 10^4$ nm), size did not influence toxicity. Aggregation of particles led to their size in suspension differing widely from that of the dry powders. The role of particle size on antibacterial activity is not clear; other variables such as surface chemistry and morphology might be more important toxicity determinants.

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 toxicity observed in dark treatments suggests that additional, as yet undetermined toxicity mechanisms might contribute to cell growth inhibition.

The toxicity levels established for TiO_2, SiO_2 and ZnO highlight the need for safe disposal protocols for each of these compounds. Their release into surface or groundwaters potentially has detrimental effects to ecosystem health.

Acknowledgements

This research was supported jointly by the Center for Biological and Environmental Nanotechnology at Rice University (EEC-0118007) and by EPA-STAR (91650901-0).

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