Implications and potential applications of bactericidal fullerene water suspensions: effect of nC_{60} concentration, exposure conditions and shelf life

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ABSTRACT

Stable fullerene water suspensions (nC_{60}) exhibited potent antibacterial activity to physiologically different bacteria in low-salts media over a wide range of exposure conditions. Antibacterial activity was observed in the presence or absence of light or oxygen, and increased with both exposure time and dose. The activity was also influenced by the nC_{60} storage conditions and by the age of the buckminsterfullerene (C_{60}) used to make nC_{60}. These results reflect the potential impact of nC_{60} on the health of aquatic ecosystems and suggest novel alternatives for disinfection and microbial control.

Key words | bacteria, fullerenes, nanomaterial, nC_{60}

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INTRODUCTION

With the current nanotechnology boom, technologies incorporating nano-scale processes and materials are being explored for the reduction of waste production, remediation of contaminant spills, water treatment, and improved energy production and usage. For example, some metal and metal-oxide nanoparticles (e.g. nanoiron, magnetite, and titanium dioxide) can serve as catalysts or reactants in the destruction of contaminants for *in situ* groundwater remediation (Liu *et al.* 1995; McCormick & Adriaens 2004; Mattigod *et al.* 2005), wastewater treatment (Ferguson *et al.* 2005; Lee *et al.* 2005), and drinking water treatment (Rincon & Pulgarin 2004) (Wei *et al.* 1994; Watts *et al.* 1995; Otaki *et al.* 2000). However, along with the potential benefits of nanotechnology, there is also the potential for adverse consequences due to the lack of risk assessment and regulation of nanomaterials. An increased understanding of the environmental impacts of nanomaterials is necessary to ensure their safe use and disposal, therefore enhancing the sustainability of the field.

As carbon-based nanomaterials, such as buckminsterfullerene (C_{60}), become increasingly available and affordable, they will potentially find widespread use in products such as cosmetics, drug delivery vectors, and semiconductors. During the production, consumption, and disposal of these products, the environmental behavior of these materials becomes relevant, specifically in aqueous based systems. While pristine C_{60} is relatively insoluble in water, it can enter the water phase through the formation of water-soluble derivatives, encapsulation by hydrophilic molecules, or the formation of stable, nanoscale water-soluble aggregates (termed here as nC_{60}). Once the molecule is stable in water, it can move away from its original source location thus increasing the media volume exposed and in the number of biological receptors. Previous research establishing the antimicrobial activity of nC_{60} indicated that several factors influence its toxicity, such as particle size, organic matter, and ionic strength of the medium (Lyon *et al.* 2005, 2006; Li *et al.* 2008). Specifically, smaller particles (with larger surface area per volume) are more toxic, soil organic matter promotes sorption that reduces bioavailability and toxicity, and higher ionic strength mitigates toxicity by promoting coagulation and precipitation.

This work evaluates additional factors that influence the antibacterial activity of nC_{60} to further evaluate potential environmental impacts and disinfection applications. Factors considered include nC_{60} concentration and time of exposure, bacterial growth conditions, the age of the fullerene and nC_{60} used, and the bacterial species tested.

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METHODS

Production and aging of nC_{60}

Fullerene powder (99.5% pure, SES Research, Houston, TX) was divided into four vials each containing 1 g, and one vial was stored under each of the following conditions: light/oxic, light/anoxic, dark/oxic, and dark/anoxic. Anoxic conditions were simulated in a Forma Scientific Anaerobic System Model 1024 (Thermo Electron Co., Marietta, OH), and dark conditions were achieved by wrapping vials in aluminium foil. From each vial of fullerene powder, 250 mL of nC_{60} were made using the method of Fortner *et al.* (2005), and each of these batches was concentrated to about 30–50 mL. The nC_{60} samples were stored under the same conditions as the fullerene powder, and both sets of samples were kept for varying time intervals to determine the effect of shell life (1 week, 6 months, or 1 year). Particle size distributions were assessed using dynamic light scattering (Brookhaven Instrument Corporation, Holtsville, NY, USA).

Bacterial growth conditions and toxicity tests

The main two bacteria used in these studies were *Escherichia coli* K12 (Gram negative) and *Bacillus subtilis* 168 (Gram positive). The minimum inhibitory concentration (MIC) of nC_{60} was determined as described earlier (Lyon *et al.* 2006). The bacteria were maintained on LB plates, but the toxicity tests were performed in a minimal Davis medium (MD) to preclude aggregation of nC_{60} (Lyon *et al.* 2006).

To assess the effect of exposure time, *E. coli* (grown overnight in LB) were added to tubes containing MD with no glucose to a final OD_{600} 0.001. They were incubated either with or without nC_{60} (2 mg/L) for up to one hour. At ten minute intervals, an aliquot of cells was diluted 1/100 in MD and 10 μL of that dilution were plated onto an LB plate. The plates were incubated overnight at 37°C, and the number of colony forming units or cfu/mL was calculated the next day. A dose response curve was constructed for *E. coli* grown in MD with nC_{60}. *E. coli* cells grown overnight in MD were diluted in fresh MD to an OD_{600} 0.001. These bacteria were diluted a thousand fold to obtain $\sim 10^4$ cfu/mL and then exposed to varying concentrations of nC_{60} for 1 hour while being shaken at 37°C. Three dilutions of each samples were plated on LB and grown overnight at 37°C. The colonies were counted the next day to calculate the concentration of colony forming units per mL (cfu/mL).

Bacteria were grown in the presence or absence of light to assess the effects of photoactivating the nC_{60}. The bacteria were grown under aerobic, fermentative, and anaerobic conditions. Due to the slow growth of the bacteria under anaerobic conditions, growth of the bacteria was assessed on MD plates. The bacteria were adjusted to their new growth condition in the anaerobic chamber prior to the test. The following types of plates were made: MD, MD + 0.2% KNO_3, MD + 0.8 mg/L nC_{60}, and MD + 0.8 mg/L nC_{60} + 0.2% KNO_3 (used as an electron acceptor under anaerobic, nitrate-reducing conditions). Plates were made by autoclaving agar (final concentration 1.6%) with MD (no glucose), transferring the hot liquid into the anaerobic chamber, adding glucose, nC_{60}, and KNO_3 to the appropriate concentration, and then pouring the plates. The plates were equilibrated for two days in the anaerobic chamber prior to use. Bacteria were spread onto the plates using glass beads and then incubated at 37°C for up to one week.

Testing different bacteria

Various bacteria were tested for susceptibility to nC_{60} using the media and growth conditions summarized in Table 1. The MIC was determined as described above.

Statistical analysis

All experiments were run at least in triplicate, and error bars (representing standard errors) are included in the figures. Where appropriate, samples were analyzed for statistical difference using Student's *t*-test at the 95% confidence interval.

RESULTS

Exposure time and dose increases toxicity

E. coli inactivation was tracked by plating the bacteria at different times after exposure. Figure 1 shows a significant decrease in the population of viable cells over time, compared

Table 1 | Different bacteria tested for susceptibility to nC_{60}

Bacteria	ATCC#	Growth medium
Bacillus subtilis 168	31578	MD
Burkholderia cepacia	10856	MD
Desulfovibrio desulfuricans	7757	ATCC 1249
Escherichia coli K12	25404	MD
Pseudomonas aeruginosa	47053	MD
Ralstonia pickettii	49129	MD
Streptomyces albus	3004	Yeast malt extract

to an nC_{60}-free control set, with a 2-log loss in viability after 50 min exposure to 2 mg/L nC_{60}. This corresponds to a 99% *Ct* value of about 100 mg-min/L, which is much higher than the 99% *Ct* value for *E. coli* disinfection reported for free chlorine (0.03–0.05 mg-min/L), but compares favorably with the corresponding value for chloramines (95–180 mg-min/L) (Hoff 1986). For additional perspective, the recommended *Ct* values for free chlorine are 20 mg-min/L to protect fish health (http://www.fws.gov/policy/aquatichandbook/Volume_3/Section_3.pdf 2005), 450 mg-min/L for tertiary treated wastewater water recycling and reuse in California (http://www.dhs.ca.gov/ps/ddwem/waterrecycling/PDFs/treatmenttechnology.pdf 2007), and 9,800 mg-min/L for fecal accidents in swimming pools (http://www.ccdeh.com/commttee/rec/guidelines/Fecal_Accidents.pdf 2001).

The dose–response curve is a common tool used in toxicology to determine the effective concentration at which 50% of the bacteria exhibit a response (EC_{50}), which in this case is loss of cell viability. For nC_{60}, the EC_{50} is around 1 mg/L (Figure 2) which is lower than the EC_{50} for the disinfectant triclosan at 5 mg/L (McDonnell & Russell 1999), indicating that nC_{60} is a more powerful antibiotic.

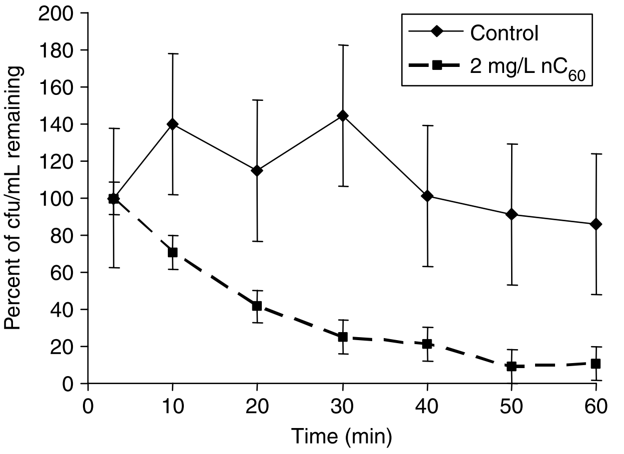

Figure 1 | Percent *E. coli* viable cells remaining after exposure to nC_{60} over the course of one hour.

Growth conditions have no effect on bacterial susceptibility to nC_{60}

For *E. coli*, the MIC values obtained in the presence of light were similar to those in the dark, at 0.01–0.05 mg/L. For *B. subtilis*, the MIC values were also similar, at 0.01–0.05 mg/L. The presence or absence of light did not affect the toxicity of the nC_{60}. *E. coli* was tested for growth with nC_{60} on MD plates under aerobic, anaerobic, and fermentative conditions while *B. subtilis* was just tested for growth under aerobic and anaerobic conditions. In all cases, the growth condition did not alter the antimicrobial activity of nC_{60}; none of the plates containing nC_{60} showed growth while all positive controls exhibited substantial growth.

Effects of storage conditions on nC_{60} antibacterial activity

The activity of nC_{60} against *E. coli* was monitored as it aged under oxic (ox) or anoxic (an) and light (lt) or dark (dk) conditions. Antibacterial activity decreased over time regardless of storage condition (Figure 3). The mean particle size of these poly-disperse suspensions was not significantly influenced by either storage conditions or age (data not shown). The activity of nC_{60} made from C_{60} stored under

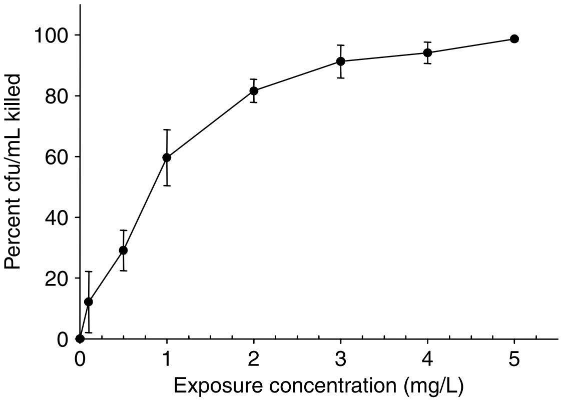

Figure 2 | Dose–response curve for *E. coli* exposed to nC_{60} for 1 h.

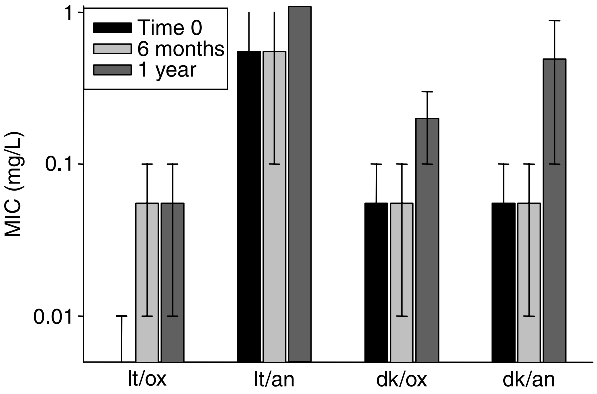

Figure 3 | MICs of nC_{60} stored under varying conditions for one week, six months, and one year. For *E. coli*, the "lt/ox 1 wk" set had an MIC < 0.01 mg/L whereas the "lt/an 6 mo" set had an MIC > 1 mg/L.

the same combination of conditions was also monitored to determine if the C_{60} itself contributed to a loss in toxicity. Aging of the C_{60} powder did not correlate to a marked increase or decrease in antibacterial activity (data not shown). However, after one year, all of the suspensions had lower antibacterial activity than the nC_{60} made with recently synthesized C_{60}.

Susceptibility of different bacteria

Different species were tested for susceptibility to nC_{60} (Table 2). All tested bacteria (Gram positive and Gram negative) were susceptible to nC_{60}. *D. desulfuricans*, an obligate anaerobe, had a higher MIC, which may be due to a higher tolerance of nC_{60} or, more likely, due to the higher ionic strength of its growth medium which promotes nC_{60} coagulation and precipitation.

Table 2 | MIC of fresh nC_{60} with different bacteria

Bacteria	Description	MIC (mg/L)
Bacillus subtilis	Gram +, soil	0.01–0.05
Burkholderia cepacia	Gram −, pathogen	0.0125–0.025
Desulfovibrio desulfuricans	Gram −, anaerobe	0.1–0.2
Escherichia coli	Gram −, potential pathogen	0.01–0.05
Pseudomonas aeruginosa	Gram −, ubiquitous pathogen	0.05–0.066
Ralstonia pickettii	Gram −, pathogen	0.025–0.0375
Streptomyces albus	Gram +, soil	<0.05

DISCUSSION

nC_{60} is a potent antibacterial agent

The antimicrobial activity of nC_{60} has been established in two previous papers (Lyon *et al.* 2005, 2006); these papers also considered the effect of certain factors, like salt concentration, which influenced antibacterial activity. Exploring these factors provides insight into the effects of nC_{60} in environmental settings, how to offset potential toxicity, and the mechanism behind nC_{60} antimicrobial activity. Assessing the antibacterial activity of nC_{60} under varying light and oxygen conditions indicates that it is a flexible antibacterial agent. Based on the ability of nC_{60} to kill bacteria under light or dark conditions, it appears that the photosensitive nature of fullerenes does not influence these observed antibacterial properties, even under anaerobic (nitrate-reducing) and fermentative conditions. The EC_{50} results reflect nC_{60} potency as an antibiotic (Figure 2), indicating potential ecological impact but also a promising antimicrobial agent in the absence of high dissolved salt concentrations or sorbents that decrease bioavailability and toxicity. This reinforces the EC_{50} and MIC data which has been previously collected (Lyon *et al.* 2005, 2006). Furthermore, while the rapidity with which nC_{60} dispatches of *E. coli* shows the potency of nC_{60} (Figure 1), the residual activity of nC_{60} has not been ascertained. The stability of nC_{60} activity over a period of a year (Figure 3) shows it may be considered for long-term disinfection applications. However, the bacterial community may be quickly impacted but is likely to recover if the nC_{60} is sorbed, precipitated or otherwise neutralized.

nC_{60} possesses antibacterial activity against a broad spectrum of bacteria (Table 2), and thus could be expected to broadly perturb microbial communities in aquatic systems and not just target certain organisms. While this is a more alarming prospect in a release scenario, it is a desirable property for an effective antimicrobial agent. Broad spectrum antibiotics are in great demand in medical, industrial, and common household settings. This broad spectrum activity also provides some clues to the antimicrobial mechanism. Certain antibiotics have very specific target organisms, and this can be linked to their *modus operandi*. For example, penicillin targets Gram positive bacteria because it is a beta lactam antibiotic, destroying the peptidoglycan layer of the cell envelope which is particularly thick in Gram positive bacteria (Prescott *et al.* 1996). There was no obvious difference between the susceptibility of Gram positive versus Gram negative bacteria. The only anaerobe tested, *D. desulfuricans*, displayed a slightly higher MIC, which could be explained by the difference in the salt content of the growth media.

The low concentrations of nC_{60} needed to exert an antibacterial effect and its increasing availability and affordability make it a potentially attractive, viable agent for wastewater and drinking water treatment. However, it is premature to recommend nC_{60} as a disinfectant before research is conducted on the scalability and competitiveness of using nC_{60} for water treatment, especially in comparison to established methods like chlorination and UV treatment, and its effects on potential ecological receptors if nC_{60} escapes disinfection reactors.

Environmental factors may lessen the microbial toxicity of nC_{60}

The versatility and potency of nC_{60} indicate potential biological disruptions in the event of exposure to soil or water ecosystems. The ability of nC_{60} to kill different types of bacteria under oxic/anoxic, light/dark conditions foreshadows the effects of nC_{60} on soil and water microbial communities. However, there are several factors which may mitigate the antibacterial activity of nC_{60}. Previous work has shown that salt concentration increased nC_{60} particle size (Lyon *et al.* 2005; Brant *et al.* 2005), and increasing particle size results in increasing MIC (decreased toxicity) (Lyon *et al.* 2006). In marine settings, the ionic strength can reach 1 M, instigating nC_{60} coagulation and a loss of toxicity either due to precipitation or an unknown factor (Bodek *et al.* 1988). In soil or water, nC_{60} may sorb to particulate matter and be immobilized or even neutralized, though the toxicity of sorbed nC_{60} has not been determined. Previous work has shown that sorption of nC_{60} to soil reduces its bioavailability and antibacterial activity, with toxicity mitigation strongly increasing with soil organic content (Li *et al.* 2008). Furthermore, nC_{60} sorbs to both *E. coli* and *B. subtilis*, with a higher propensity for *E. coli*. Along with reports of the slow movement of nC_{60} in porous media (Lecoanet *et al.* 2004), these results suggest that nC_{60} will not disperse widely in the environment, and its antibacterial activity will be diminished by sorption and aggregation processes. A recent study examining soil health after exposure to C_{60} and nC_{60} found no marked difference (Tong *et al.* 2007).

Both fullerenes and nC_{60} were aged to determine the effect of shelf life on the antibacterial activity of nC_{60}. nC_{60} particles are known to be stable for at least several months (Brant *et al.* 2005). However, nC_{60} stored under any conditions decreased in toxicity over time. We investigated whether the decrease in toxicity was due to an increase in particle size, which has been reported to increase with storage time (Brant *et al.* 2005). In this case, however, the mean particle diameter of the different suspensions only varied from 81–125 nm, and there was no obvious correlation with changes in toxicity.

The fullerene powder used to make nC_{60} was also aged under different storage conditions. The nC_{60} made with 1-year old C_{60} appears to have slightly less antibacterial activity than nC_{60} made with fresh C_{60}. This may be due to C_{60} oxidation upon exposure to air under ambient conditions as reported by others (Huffman & Ganske 1995). It has been shown previously that the hydroxylated fullerenes that would be formed by this oxidation have no discernible antibacterial activity (Lyon *et al.* 2005).

CONCLUSIONS

nC_{60} is a strong broad spectrum antibacterial agent, able to maintain its toxicity under varying environmental conditions with significant potential as an antibacterial agent for water treatment and biofouling control. Despite its effectiveness, several factors have the potential to pacify nC_{60} in environmental systems, such as high ionic strength, sorption, and aging. Further research on the environmental impacts of nanomaterials in the early stages of nanotechnology development is recommended to enhance risk assessment, effective regulation, and better public acceptance.

REFERENCES

Bodek, I., Lyman, W. J., Reehl, W. F. & Rosenblatt, D. H. (Eds) 1988 *Environmental Inorganic Chemistry: Properties, Processes, and Estimation Methods*. Pergamon Press, New York.

Brant, J., Lecoanet, H. & Wiesner, M. R. 2005 Aggregation and deposition characteristics of fullerene nanoparticles in aqueous systems. *J. Nanoparticle Res.* **7**, 545–553.

Ferguson, M. A., Hoffmann, M. R. & Hering, J. G. 2005 TiO_2^-photocatalyzed As(II) oxidation in aqueous suspensions: reaction kinetics and effects of adsorption. *Environ. Sci. Technol.* **39**, 1880–1886.

Fortner, J. D., Lyon, D. Y., Sayes, C. M., Boyd, A. M., Falkner, J. C., Hotze, E. M., Alemany, L. B., Tao, Y. J., Guo, W., Ausman, K. D., Colvin, V. L. & Hughes, J. B. 2005 C_{60} in water: nanocrystal formation and microbial response. *Environ. Sci. Technol.* **39**, 4307–4316.

Hoff, J. C. 1986 *Inactivation of microbial agents by chemical disinfectants*, EPA/600/S602-686/067, US Environmental Protection Agency, Cincinnati, OH, USA.

Huffman, A. M. & Ganske, J. A. 1995 Characterization of fullerene materials and their oxidative stability using diffuse reflectance infrared fourier transform spectroscopy. *Appl. Spectrosc.* **49**, 534–537.

http://www.ccdeh.com/commttee/rec/guidelines/Fecal_Accidents.pdf 2001 California Conference of Directors of Environmental Health, Recreational Health Committee.

http://www.dhs.ca.gov/ps/ddwem/waterrecycling/PDFs/treatmenttechnology.pdf 2007 State of California, Department of Health Services Division of Drinking Water and Environmental Management.

http://www.fws.gov/policy/aquatichandbook/Volume_3/Section_3.pdf 2005 U.S. Fish and Wildlife Service, Washington, D.C.

Lecoanet, H. F., Bottero, J.-Y. & Wiesner, M. R. 2004 Laboratory assessment of the mobility of nanomaterials in porous media. *Environ. Sci. Technol.* **38**, 5164–5169.

Lee, J., Choi, W. & Yoon, J. 2005 Photocatalytic degradation of N-nitrosodimethylamine: mechanism, product distribution, and TiO_2 surface modification. *Environ. Sci. Technol.* **39**, 6800–6807.

Li, D., Lyon, D. Y., Li, Q. & Alvarez, P. J. J. 2008 Effect of natural organic matter on antibacterial activity of a fullerene water suspension. *Environ. Toxicol. Chem.* (Submitted).

Liu, D., Johnson, P. R. & Elimelech, M. 1995 Colloid deposition dynamics in flow through porous media: role of electrolyte concentration. *Environ. Sci. Technol.* **29**, 2963–2973.

Lyon, D. Y., Adams, L. K., Falkner, J. C. & Alvarez, P. J. J. 2006 Antibacterial activity of fullerene water suspensions: effects of preparation method and particle size. *Environ. Sci. Technol.* **40**, 4360–4366.

Lyon, D. Y., Fortner, J. D., Sayes, C. M., Colvin, V. L. & Hughes, J. B. 2005 Bacterial cell association and antimicrobial activity of a C_{60} water suspension. *Environ. Toxicol. Chem.* **24**, 2757–2762.

Mattigod, S. V., Fryxell, G. E., Alford, K., Gilmore, T., Parker, K., Serne, J. & Engelhard, M. 2005 Functionalized TiO_2 nanoparticles for use for in situ anion immobilization. *Environ. Sci. Technol.* **39**, 7306–7310.

McCormick, M. L. & Adriaens, P. 2004 Carbon tetrachloride transformation on the surface of nanoscale biogenic magnetite particles. *Environ. Sci. Technol.* **38**, 1045–1053.

McDonnell, G. & Russell, A. D. 1999 Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.* **12**, 147–179.

Otaki, M., Hirata, T. & Ohgaki, S. 2000 Aqueous microorganisms inactivation by photocatalytic reaction. *Water Sci. Technol.* **42**(3-4), 103–108.

Prescott, L. M., Harley, J. P. & Klein, D. A. 1996 *Microbiology*. Wm. C. Brown Publishers, Chicago.

Rincon, A. & Pulgarin, C. 2004 Effect of pH, inorganic ions, organic matter and H_2O_2 on *E. coli* K12 photocatalytic inactivation by TiO_2: Implications in solar water disinfection. *Appl. Catal. B: Environ.* **51**, 283–302.

Tong, Z., Bischoff, M., Nies, L., Applegate, B. & Turco, R. F. 2007 Impact of fullerene (C_{60}) on a soil microbial community. *Environ. Sci. Technol.*, **41**, 2985–2991.

Watts, R. J., Kong, S., Orr, M. P., Miller, G. C. & Henry, B. E. 1995 Photocatalytic inactivation of coliform bacteria and viruses in secondary wastewater effluent. *Water Res.* **29**, 95–100.

Wei, C., Lin, W. Y., Zainal, Z., Williams, N. E., Zhu, K., Kruzic, A. P., Smith, R. L. & Rajeshwar, K. 1994 Bactericidal activity of TiO_2 photocatalyst in aqueous media: toward a solar-assisted water disinfection system. *Environ. Sci. Technol.* **28**, 934–938.