

Implications and potential applications of bactericidal fullerene water suspensions: effect of nC₆₀ concentration, exposure conditions and shelf life

D. Y. Lyon, D. A. Brown and P. J. J. Alvarez

ABSTRACT

Stable fullerene water suspensions (nC₆₀) 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 nC₆₀ storage conditions and by the age of the buckminsterfullerene (C₆₀) used to make nC₆₀. These results reflect the potential impact of nC₆₀ on the health of aquatic ecosystems and suggest novel alternatives for disinfection and microbial control.

Key words | bacteria, fullerenes, nanomaterial, nC₆₀

D. Y. Lyon
D. A. Brown
P. J. J. Alvarez
Department of Civil and Environmental
Engineering,
Rice University,
Houston, TX 77005,
USA
E-mail: alvarez@rice.edu

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 (C₆₀), 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 C₆₀ 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 nC₆₀). 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 nC₆₀ 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 nC₆₀ to further evaluate potential environmental impacts and disinfection applications. Factors considered include nC₆₀ concentration and time of exposure, bacterial growth conditions, the age of the fullerene and nC₆₀ used, and the bacterial species tested.

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METHODS

Production and aging of nC₆₀

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 nC₆₀ were made using the method of Fortner *et al.* (2005), and each of these batches was concentrated to about 30–50 mL. The nC₆₀ 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 shelf 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 nC₆₀ 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 nC₆₀ (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 OD₆₀₀ 0.001. They were incubated either with or without nC₆₀ (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 nC₆₀. *E. coli* cells grown overnight in MD were diluted in fresh MD to an OD₆₀₀ 0.001. These bacteria were diluted a thousand fold to obtain ~10⁴ cfu/mL and then exposed to varying concentrations of nC₆₀ 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 nC₆₀. 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% KNO₃, MD + 0.8 mg/L nC₆₀, and MD + 0.8 mg/L nC₆₀ + 0.2% KNO₃ (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, nC₆₀, and KNO₃ 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 nC₆₀ 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

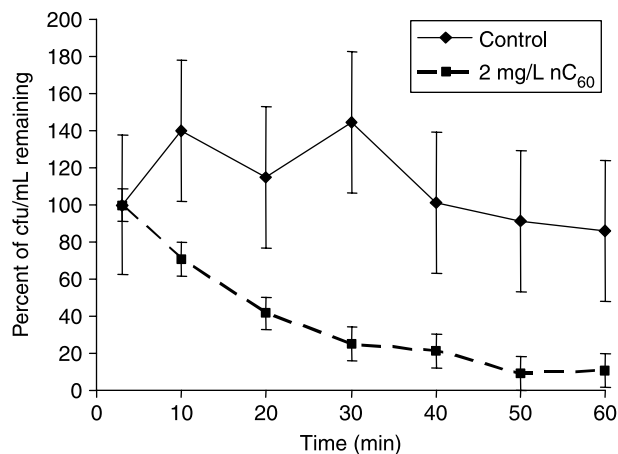
E. coli inactivation was tracked by plating the bacteria at different times after exposure. Figure 1 shows a significant decrease in the population of viable cells over time, compared

Table 1 | Different bacteria tested for susceptibility to nC₆₀

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

to an nC₆₀-free control set, with a 2-log loss in viability after 50 min exposure to 2 mg/L nC₆₀. 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.cddeh.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

**Figure 1** | Percent *E. coli* viable cells remaining after exposure to nC₆₀ over the course of one hour.

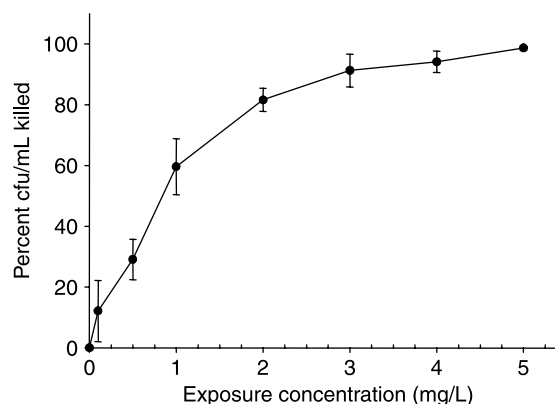
50% of the bacteria exhibit a response (EC₅₀), which in this case is loss of cell viability. For nC₆₀, the EC₅₀ is around 1 mg/L (Figure 2) which is lower than the EC₅₀ for the disinfectant triclosan at 5 mg/L (McDonnell & Russell 1999), indicating that nC₆₀ is a more powerful antibiotic.

Growth conditions have no effect on bacterial susceptibility to nC₆₀

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 nC₆₀. *E. coli* was tested for growth with nC₆₀ 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 nC₆₀; none of the plates containing nC₆₀ showed growth while all positive controls exhibited substantial growth.

Effects of storage conditions on nC₆₀ antibacterial activity

The activity of nC₆₀ 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 nC₆₀ made from C₆₀ stored under

**Figure 2** | Dose–response curve for *E. coli* exposed to nC₆₀ for 1 h.

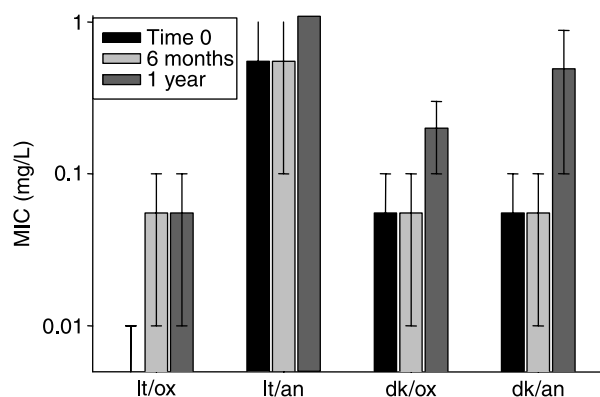


Figure 3 | MICs of nC₆₀ stored under varying conditions for one week, six months, and one year. For *E. coli*, the "lt/ox 1 wk" set had an MIC < 0.01 mg/L whereas the "lt/an 6 mo" set had an MIC > 1 mg/L.

the same combination of conditions was also monitored to determine if the C₆₀ itself contributed to a loss in toxicity. Aging of the C₆₀ 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 nC₆₀ made with recently synthesized C₆₀.

Susceptibility of different bacteria

Different species were tested for susceptibility to nC₆₀ (Table 2). All tested bacteria (Gram positive and Gram negative) were susceptible to nC₆₀. *D. desulfuricans*, an obligate anaerobe, had a higher MIC, which may be due to a higher tolerance of nC₆₀ or, more likely, due to the higher

Table 2 | MIC of fresh nC₆₀ with different bacteria

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

ionic strength of its growth medium which promotes nC₆₀ coagulation and precipitation.

DISCUSSION

nC₆₀ is a potent antibacterial agent

The antimicrobial activity of nC₆₀ 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 nC₆₀ in environmental settings, how to offset potential toxicity, and the mechanism behind nC₆₀ antimicrobial activity. Assessing the antibacterial activity of nC₆₀ under varying light and oxygen conditions indicates that it is a flexible antibacterial agent. Based on the ability of nC₆₀ 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 EC₅₀ results reflect nC₆₀ 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 EC₅₀ and MIC data which has been previously collected (Lyon *et al.* 2005, 2006). Furthermore, while the rapidity with which nC₆₀ dispatches of *E. coli* shows the potency of nC₆₀ (Figure 1), the residual activity of nC₆₀ has not been ascertained. The stability of nC₆₀ 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 nC₆₀ is sorbed, precipitated or otherwise neutralized.

nC₆₀ 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 nC₆₀ 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 nC₆₀ as a disinfectant before research is conducted on the scalability and competitiveness of using nC₆₀ for water treatment, especially in comparison to established methods like chlorination and UV treatment, and its effects on potential ecological receptors if nC₆₀ escapes disinfection reactors.

Environmental factors may lessen the microbial toxicity of nC₆₀

The versatility and potency of nC₆₀ indicate potential biological disruptions in the event of exposure to soil or water ecosystems. The ability of nC₆₀ to kill different types of bacteria under oxic/anoxic, light/dark conditions foreshadows the effects of nC₆₀ on soil and water microbial communities. However, there are several factors which may mitigate the antibacterial activity of nC₆₀. Previous work has shown that salt concentration increased nC₆₀ 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 nC₆₀ coagulation and a loss of toxicity either due to precipitation or an unknown factor (Bodek *et al.* 1988). In soil or water, nC₆₀ may sorb to particulate matter and be immobilized or even neutralized, though the toxicity of sorbed nC₆₀ has not been determined. Previous work has shown that sorption of nC₆₀ to soil reduces its bioavailability and antibacterial activity, with toxicity

mitigation strongly increasing with soil organic content (Li *et al.* 2008). Furthermore, nC₆₀ sorbs to both *E. coli* and *B. subtilis*, with a higher propensity for *E. coli*. Along with reports of the slow movement of nC₆₀ in porous media (Lecoanet *et al.* 2004), these results suggest that nC₆₀ 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 C₆₀ and nC₆₀ found no marked difference (Tong *et al.* 2007).

Both fullerenes and nC₆₀ were aged to determine the effect of shelf life on the antibacterial activity of nC₆₀. nC₆₀ particles are known to be stable for at least several months (Brant *et al.* 2005). However, nC₆₀ 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 nC₆₀ was also aged under different storage conditions. The nC₆₀ made with 1-year old C₆₀ appears to have slightly less antibacterial activity than nC₆₀ made with fresh C₆₀. This may be due to C₆₀ 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

nC₆₀ 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 nC₆₀ 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.

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