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CRITICAL REVIEW

The antibacterial effects of engineered nanomaterials: implications for wastewater treatment plants†

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Nanotechnology is currently at the forefront of scientific research and technological developments that have resulted in the manufacture of novel consumer products and numerous industrial applications using engineered nanomaterials (ENMs). With the increasing number of applications and uses of ENMs comes an increasing likelihood of nanoscale materials posing potential risks to the environment and engineered technical systems such as wastewater treatment plants (WWTPs). Recent scientific data suggests that ENMs that are useful in, for example, medical applications due to their novel physicochemical properties, may also cause adverse effects to the bacterial populations used in wastewater treatment systems. In this review, the toxicological effects of titanium nanoparticles ($nTiO_2$), zinc oxide (nZnO), carbon nanotubes (CNTs), fullerenes (C_{60}) and silver nanoparticles (AgNPs) to bacteria were examined. The results suggest that the potential ENMs risks to bacteria are non-uniform (need to be assessed case-by-case), and are dependent on numerous factors (*e.g.* size, pH, surface area, natural organic matter). Currently available data are therefore insufficient for evaluating the risks that ENMs pose in WWTPs. To fill these knowledge gaps, we recommend scenario specific studies aimed at improving our understanding on: (i) estimated volumes of ENMs in effluents, (ii) the antibacterial sensitivity of cultures within WWTPs towards selected ENMs, and (iii) processes improving the stability of ENMs in solutions. Two factors that merit consideration for elucidating the potential risks systematically are the toxicity mechanisms of ENMs to bacteria, and the influencing factors based on inherent physicochemical properties and environmental factors. Furthermore, the complexity of behaviour and fate of ENMs in real WWTPs requires case studies for assessing the ENMs risks to bacteria *in vivo*. The current laboratory results derived using simplified exposure media do not reflect actual environmental conditions.

1. Introduction

1.1. Nanotechnology and engineered nanomaterials

Nanotechnology-driven capabilities due to recent technological advancements have offered novel opportunities for manufacturing nanoscale materials—generically referred to as engineered nanomaterials (ENMs). This has led to wide applications of ENMs and production of nanotechnology-enabled products (nanoproducts) which have begun to shape the global

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Environmental impact

This review critically evaluates the existing knowledge on engineered nanomaterials (ENMs) as a potential threat to wastewater treatment plants (WWTPs) through the evaluation of their behaviour, fate, and mechanisms of toxicity to the bacteria populations. The antibacterial effects of ENMs depend on physicochemical properties and environmental factors, which vary depending on the type of ENMs, exposure conditions and type of bacteria. Using available data, the review concludes that ENMs may potentially have adverse effects on the treatment efficiency of WWTPs. Focused research studies that take into account actual environmental conditions, are necessary for advancing our ability to assess these risks.

economy through commercialization of consumer products (*e.g.* cosmetics, medicines, drugs, *etc.*) as well as in environmental remediation and industrial applications.

For example, the number of nanoproducts in the inventory of the Woodrow Wilson International Centre for Scholars[1] increased from 54 in March 2005 to 1015 in August 2009. The Nanowerk Nanomaterial Database Inventory[2] listed 1979 products in August 2008, and 2238 in May 2009. The majority of products were in the categories of single metals (*e.g.* silver, zinc or titanium), or binary compounds and fullerenes. The global production statistics of ENMs suggest rapidly increasing trends since 2000, as indicated in Table 1.

Given the large diversity of ENMs being manufactured by many companies in different countries[1]—and the increasing research interest in the field of nanotechnology as evidenced by more than 80 000 journal articles by the year 2009[12]—the potential risks of ENMs after their release into the environment are a growing concern. In this review, the authors highlight the effects of ENMs namely; AgNPs, nZnO, $nTiO_2$, CNTs, and fullerenes on microbial communities—and how they may impair the function of wastewater treatment plants (WWTPs). The choice of these ENM types is based on a recent analysis of nanoproducts which suggested that the most common products contain carbon based- (fullerenes, CNTs), metal based- (silver), and metal oxide based- ($nTiO_2$, nZnO) materials.[1,13] These ENMs are therefore likely to be primary candidates for current and immediate release into the environment in large volumes. Lastly, the amount of information in the scientific literature on the antibacterial properties of ENMs has increased, providing data and knowledge useful in elucidating the potential risk they may pose to microbial populations in WWTPs.

Table 1 Global production statistics of engineered nanomaterials reported in tonnes per annum

ENMs type/year	2002	2003	2004	2005	2006	2007	2008	Reference
$nTiO_2$						5000	60 926	3,4
AgNP				4			563	5,6
CNT			473		500	278	140	4,7–9
nZnO						20	9845	3,4
C_{60}	0.15			10				10,11

The growth in the nanotechnology industry and the types and applications of ENMs are leading to an increase in the release of these nanoscale materials into the environment in significant quantities. ENMs are by definition in the nanoscale range, so they posses unique physicochemical properties which determine their fate and behaviour in the environment, their distribution and their toxicological effects to biological systems, which probably differs markedly from those of their counterpart bulk parent materials. Some ENMs, mostly from nanowaste streams,[13,14] will enter WWTPs, thereby potentially posing a risk to the bacteria that facilitate the treatment of effluent and sludge, and that uphold the integrity of natural ecosystems. By compromising the bacterial ecology of WWTPs, not only do they compromise treatment efficiency of conventional micro-pollutants such as inorganics, metals, organics and endocrine disrupting chemicals, but also the integrity of receiving natural aquatic ecosystems.

1.2. ENMs in wastewater treatment systems

During the production, use, and disposal phases of ENMs life-cycle: they will inevitably be released into the environment, and their concentrations are increasing year by year.[13–21] The most probable exposure route of ENMs to the environment is through

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the release of effluents from domestic and industrial applications into wastewaters and surface waters.[13,14,16,20] Consequently, the ENMs are likely to interact with useful bacteria populations during wastewater treatment processes or after the application of the biosolids onto the agricultural soils as fertilizer (sourced from WWTPs).

The predicted environmental exposures of ENMs derived using modelling techniques[6,14–18] reported to date are supported by the detection of ENMs from nanoproducts at usage or through disposal phases in wastewater systems.[22–27] An early report quantified the $nTiO_2$ and bulk Ti in a WWTP—with a maximum Ti value of up to 2.8 mg L^{-1} (average 0.84 mg L^{-1}) in influent water and 8.5 mg L^{-1} reported in secondary solids (sludge). The treated effluent concentrations of ENMs ranged from 0.001 to 0.1 mg L^{-1}, suggesting their high affinity for sludge biosolids.[25]

Different sizes (<50 nm to <70 μm) and aggregation states of TiO_x, including $nTiO_2$, occur at various treatment stages, as confirmed using EDX and SEM imagery analysis techniques.[25] Other studies have suggested that $nTiO_2$ and SiO_2 are likely to separate from nano-composites during usage.[19] AgNPs from antimicrobial coatings and composites could also find their way into aquatic environments from agricultural and food nanotechnology-based applications.[28]

$nTiO_2$ has been detected in runoff water from exterior walls of a building in an urban area in different sizes (<10 nm to >150 nm) and aggregation states,[24] and similar sized particles have been detected on walls and urban surface runoff.[25] Kim and coworkers[26] detected and characterised nAg_2S particles (5–20 nm) in treated sludge from a WWTP formed due to the reactions of H_2S and AgNPs under anaerobic conditions. However, due to the complexity of physicochemical and biological water parameters in natural and man made water courses including WWTPs, the current analytical techniques are limited for quantifying ENMs in water, solid, and biological samples.[29–31]

Fullerenes have recently been detected in wastewater using high-performance liquid chromatography (HPLC).[32] The amounts ranged from 0.2 to 1 ng L^{-1} as suspended solids in effluents from WWTPs. Of the three compounds analysed, C_{60}, C_{70} fullerenes and *N*-methylfulleropyrrolidine C_{60}, the C_{60} fullerenes were found as most abundant.[31]

Table 2 Sample of modelled quantities of ENMs in effluents for several different regions globally

	SW[a] (Muller and Nowack, 2008)[16]		Gottschalk *et al.*, 2009[b17]			SA[c] (Musee, 2010)[14]	
ENM[d]	RE	HE	EU Mode	US Mode	SW Mode	Pro	Max
$nTiO_2$	0.7	16	3.47	1.75	4.28	0.041	0.270
nAg	0.03	0.08	42.5	21.0	38.7	0.043	0.620
CNT	0.0005	0.0008	14.8	8.6	11.8	—	—
nC_{60}	—	—	5.2	4.6	3.82	—	—
nZnO	—	—	0.432	0.3	0.441	—	—

[a] RE: realistic scenario, HE: high emission scenario. [b] Mode: most frequent value. [c] Pro: probable scenario, Max: maximum scenario. [d] The concentrations are expressed in μg L^{-1}. SW: Switzerland, SA: South Africa

Reviews and studies have noted the difficulty of analytical quantification of ENMs in actual environmental compartments,[24,29,33,34] which explains why few studies of this nature have been published. The limitations of analytical quantification have encouraged modelling approaches to provide quantification estimates of ENMs in wastewater and environmental media[16–18,20,21] as shown in Table 2. The increasing use of ENMs coupled with the lack of risk assessment data concerning their fate, behaviour and toxicity in biological systems,[8] has motivated ecotoxicity research on the effects of ENMs on various microbial communities. This paper reviews the scientific knowledge and trends of the effects of ENMs on bacteria and the environmental significance of this, with special focus on bacterial communities in WWTPs. An understanding of the adverse effects of ENMs due to their antibacterial properties[35–38] is important because of their potential to disrupt bacterial populations that perform vital functions, for example, the degradation of organic matter, transformation of elements, and recycling of nutrients.[39,40]

1.3. ENMs stability in wastewater treatment systems

The stability of ENMs in aquatic media, including engineered systems like WWTPs, influences their fate, behaviour, and toxicity to microbial communities. Many types and forms of ENMs are insoluble, so their degree of stability in the WWTPs largely determines the severity of observed toxicological effects on the receptor bacterium. Handy *et al.*[34] discussed the influence of chemistry and environmental factors with reference to the observed toxicological effects on the receptor organisms. The stability of ENMs in aquatic environments depends on numerous factors such as: (i) particle shape, size, surface area, and surface charge on the aggregation chemistry; (ii) aggregation and the ability to form stable dispersions in aqueous systems, (iii) adsorption of ENMs onto surfaces, including the exterior surfaces of organisms; and (iv) abiotic factors such as pH, ionic strength (salinity), water-hardness, natural organic matter, and other chemicals in the environment.

Data on the stability of ENMs in WWTPs, or even in aquatic systems in general, is largely lacking. Therefore research attention is needed towards elucidating mechanisms that control the stability of ENMs in water, and how that affects the potential fate, behaviour and toxicity of ENMs, particularly to the microbial communities in WWTPs. The toxicological effects of ENMs are closely linked to their colloidal stability, which is the single most important factor influencing their bioavailability to aquatic biota.[36,41,42] The stability of ENMs in water is a function of their solubility and dispersibility, which in turn controls the degree of ENM aggregation after they enter aquatic systems and ultimately their potential to cause observable toxicological effects on receptor organisms.

The sorption of nC_{60} into the soil due to the presence of organic matter has been shown to attenuate its bioavailability,[43] hence reducing the antibacterial activity. For example, adsorption of dissolved humic substances onto nC_{60} appeared to attenuate its antibacterial activity even at humic acid concentrations as low as 0.05 mg L^{-1},[43] because the natural organic matter (NOM) on nC_{60} prevented direct contact of nC_{60} with bacteria cells. Alternatively, the NOM may have reacted with nC_{60}, thereby promoting its disaggregation, or changing its

surface chemistry and consequently reducing the antibacterial activity. The possibility exists that both mechanisms occur concurrently, so that the observed antibacterial toxicity effects are synergistic rather than sequential.

Brunner *et al.*[44] suggested that the solubility of oxide ENMs strongly influences their cytotoxicity where highly soluble compounds like nZnO exhibited elevated toxicity towards mammalian cells *in vitro* in comparison with less soluble nanoparticles such as $nTiO_2$. Similarly, Zhu *et al.*[45] showed that higher solubility of nZnO in comparison to other metal oxides ENPs of $nTiO_2$ and nAl_2O_3 accounted for elevated 96 h acute toxicity on zebrafish embryos. These results suggest that the inherent stability of ENMs is an important factor affecting their fate in the environment and potential biological interactions and effects. Other findings[46] suggested that the high ionic strength of divalent electrolytes destabilises ENMs, and reduces their absolute zeta potentials.

Auffan *et al.*[42] suggested that the redox properties and solubility of metallic ENMs in biological media may aid in predicting their toxicity. For example, chemically stable metallic ENMs in physiological redox conditions appeared not to exhibit cytotoxicity *in vitro* (*e.g.* gFe_2O_3 ENMs), whereas metallic ENMs with strong oxidant power (*e.g.* CeO_2, Mn_3O_4 and Co_3O_4 ENMs) or reductive power (*e.g.* FeO, Fe_3O_4, Ag^0 and Cu^0 ENMs) were cytotoxic and genotoxic towards biological targets *in vitro*.[42]

The nZnO ENMs appear to be more toxic to *E. coli* than Fe_2O_3, Y_2O_3, TiO_2, and CuO metal oxide ENMs.[47] One plausible explanation for the observed toxicity is that nZnO is an excellent photocatalyst characterised by a high dissolution rate in comparison to other forms of ENMs, in which free electrons and holes could be generated by light stronger than its band gap energy. The electron–hole pairs had the ability to diffuse out to the surface and transform the surrounding oxygen or water molecules into hydroxyl radicals *via* strong oxidation.[48]

Another plausible influencing factor on the stability of ENMs is surface chemistry.[49] For example, cytotoxicity, genotoxicity, and the ability to generate reactive oxygen species (ROS) were assessed for nZnO by varying its surface chemistry through functionalization using oleic acid (OA), poly(methacrylic acid) (PMAA), or components adsorbed from cell culture medium (medium-soaked). Uncoated ENMs showed ROS accumulation and diminished cell viability, whereas all tested surface coatings aided in the reduction of ROS production and cytotoxicity. The ability of coatings to reduce the cytotoxicity of nZnO was ranked in the following order: medium-soaked $\approx$ PMAA > OA, *i.e.* the lowest toxicity was achieved with a surface coating of components using a cell culture medium.[49]

A comparative study on the toxicity of metal oxide (TiO_2, Al_2O_3, ZnO, and SiO_2) ENMs[50] to three model bacteria species, namely gram positive *Bacillus subtilis*, gram-negative *Escherichia coli* and *Pseudomonus fluorescens* revealed nZnO as most toxic with the LC_{100} at 20 mg L^{-1}. Again, the elevated bacterial toxicity of nZnO was attributed to its high solubility. The studies suggest that the stability of ENMs in aquatic systems, including WWTPs, is an important contributor to their effect on microbial communities.

Li and colleagues[51] reported ability of ozone to oxidize SWCNT (O-SWCNT) and consequently reduce the particle size, resulting in an increase of the O-SWCNT stability in suspension during a 60 day period. However, the studies were carried out in pure water suspension media, with no or extremely low concentrations of electrolytes, which poorly represented the actual kinetic dynamics of ENMs in WWTPs. The presence of simple electrolytes and humic acid greatly enhances the aggregation kinetics[52,53] and ultimately the stability of ENMs. The findings suggest the aggregation behaviour and stability of ENMs in WWTPs are likely to vary considerably in comparison to those observed in synthetic solutions used in the laboratory studies. In summary, based on the available scientific literature, the stability of different ENMs in WWTPs is poorly understood, and the available data are inadequate to allow generalized deductions supporting risk assessment of ENMs to the microbial communities.

Therefore the aims of this paper are to: (i) examine the potential threats of ENMs to the microbial populations in natural and engineered systems (*e.g.* WWTPs) based on published toxicological data of chemicals with nanoscale dimensions; (ii) identify a set of parameters that can be useful in setting benchmarks for monitoring the behaviour and effects of ENMs in the environment due to their antibacterial properties; and (iii) provide a summary of mechanisms and factors that influence the antibacterial activity of ENMs, and how this knowledge can be exploited in developing mitigating measures that safeguards the integrity of WWTPs efficiency and reduce the adverse effects of ENMs to the receiving environment.

2. Role of biological treatment of wastewater in WWTPs

The amount of oxygen required by microorganisms to oxidise dissolved and suspended organic matter is the biological oxygen demand (BOD).[54] Municipal and industrial wastewaters contain high volumes of organic matter resulting in high BOD concentrations. Oxygen deprivation of water, especially in natural resources, gives rise to anaerobic conditions that *suffocate* all aerobic organisms, with adverse ecological effects. Municipal wastewater has a BOD of about 200 while industrial effluents can be as high as 1500 BOD units—yet efficiently treated effluent should have a BOD of less than 5 BOD units.[55] Treatment processes which reduce the quantity of BOD in the effluent and various other forms of micropollutants utilise many forms of biological manipulation, with bacteria being the most common microorganisms used.

The use of bacteria and various microorganisms to remove pollutants in wastewater is an established method for treating industrial and municipal wastewater effluents. The approach relies on the ability of bacteria to utilise a variety of wastewater chemical contaminants in their metabolic activities, resulting in the removal or reduction of contaminants concentrations in the wastewater. Bacterial-based treatment processes offer several benefits towards maximizing plant treatment efficiency, including low cost, the ability to transform a wide variety of contaminants and reduce their concentrations, potential to completely remove pollutants, including persistent organic contaminants and inorganic compounds (thereby reducing effluent toxicity) and the ability to function in the rapidly fluctuating physical and chemical conditions in wastewater.[39]

Depending on the wastewater quality and discharge quality specifications, the modern WWTP exploits the combined capabilities of different types of microbiological treatment (anoxic, aerobic, and anaerobic) to offer the highest possible treatment efficiency relative to chemical and physical processes that are generally costly, laborious, and time consuming. In a variety of WWTP types, microorganisms (mostly bacteria) are dominant and are responsible for numerous pollutant degradation reactions. Therefore, the performance and efficiency of a WWTP greatly depends on the composition and health of the microbial community.[56] Microbiological treatment approaches do not completely replace physical and chemical forms of effluent treatments, however, are widely utilised due to their suitability in certain treatment steps. In the following sections, different types of bacteria species and the targeted chemical micropollutants for removal from the wastewater are summarized.

2.1. Inorganic substance removal

Chemicals containing nitrogen and phosphate often occur in high volumes in wastewater. The complete or partial removal of such chemical constituents is necessary before discharge of the effluent into the environment, to avoid aquatic toxicological effects and eutrophication. Anaerobic and aerobic biological processes combined can reduce or even completely remove growth nutrients.[56] Phosphorus removal is often achieved through a process called enhanced biological phosphorus removal (EBPR) which runs activated sludge through anaerobic and aerobic conditions.

Under anaerobic conditions, the phosphorus is released by the hydrolysis of polyphosphate and utilised for fatty acids uptake. However, during aerobic conditions several specialised bacteria replenish their polyphosphate reserves through aerobic uptake of phosphorus from sludge.[56] Polyphosphate- and glycogen-accumulating bacteria have the ability to accumulate intracellular polyphosphates, and this capability is manipulated through various chemical, physical and biotechnological tools to remove phosphorus from the WWTPs.[57] The microbial removal of nitrogen in wastewater treatment plants (WWTP) consists of three stages, *viz*.: nitrification, denitrification and anaerobic oxidation of ammonium.[58] Numerous bacteria species from various phylogenetic classes are used in the elimination of nitrogen and phosphates, for example, the *Acinobacter*, *Betaproteobacter*, *Nitrosomonas*, *Nitrobacter*, *Nitrospira*, *Gemmatimona*, and *Thiosphaera*.[56,58,59]

2.2. Metal ion removal

Elevated concentrations of metals are often present in wastewaters, especially those from industrial and mining sources. Although most trace and heavy metals are essential for metabolism, elevated concentrations can be toxic to aquatic organisms. Therefore, it is important for the WWTPs to reduce heavy metal content to acceptable levels from wastewater before release into the environment, or treat the sludge before its use in agricultural applications. In wastewater treatment systems bacteria remove metal ions by altering the metal ion redox state, biosorption, or bioaccumulation.[59–61]

The use of metal ions as electron receptors during anaerobic respiration is one route of removing metal ions from wastewater. Examples of ion alteration or removal include the reduction of Hg^{+} to zerovalent Hg using *Escherichia coli* and *Thiobacillus ferrooxidans*.[62] The bacteria also play an important role in the bioremediation of radionuclides through the alteration of the metal ions toxicity.[63] The treatment of mining effluents containing high metal content relies heavily on the utilisation of bacteria to lower the metal content or to change the metal ion composition in the wastewater. A wide spectrum of bacterial groups are used in various treatment steps of metal ion removal process and are sometimes coupled with either physical or chemical manipulation such as changing the pH in order to alter speciation and increase metal bioavailability.

2.3. Organic contaminant removal

Organic xenobiotics such as dyes, pesticides, fuels, antibiotics, solvents and chlorinated phenolics are among the most challenging contaminants to reduce and remove during treatment of wastewater and sludge in municipal and industrial WWTPs. An array of organic compounds entering effluents, such as pesticides, pharmaceuticals and personal care products are by design expected to alter biological functions, and are known to have toxic, mutagenic, carcinogenic and teratogenic properties characterized by persistency, hydrophobicity, and lipophillicity. Generally, organic compounds reaching WWTPs are highly undesirable in the receiving environment (aquatic and terrestrial ecosystems), which in later stages can be a direct or indirect source of drinking water for humans and livestock, or for irrigating crops.

Therefore, bacteria in WWTPs are used to partially or completely degrade organic compounds through aerobic or anaerobic processes. Furthermore, partially degraded products can also be utilised as substrates for other bacterial decomposition pathways[59] or can be further degraded chemically. Sinha *et al.*[64] listed 32 bacterial genera capable of degrading organic compounds like pesticides, halogenated organic compounds, PAH compounds, phthalates, PCBs, dioxins, and petroleum products. Examples of such bacteria include; *Pseudomonas*, *Mycobacterium*, *Arthrobacter*, *Acinobacter* and *Bacillus* species. In the current scientific literature, the role of bacteria in removing various forms of persistent organic chemicals, such as halogenated phenols,[65,66] pharmaceuticals,[67,68] aromatic compounds,[69,70] dibenzofurans and dioxins[71–73] have been well documented, and the trends have been summarized in several recent reviews.[59,74–77]

2.4. Endocrine disrupting chemical removal

There is rapidly growing global concern and awareness regarding increasing exposure to endocrine disrupting chemicals (EDCs) as well as accumulative scientific knowledge quantifying the multiple effects of such chemicals on diverse biological systems including humans. EDCs in wastewaters, especially municipal wastewater are a well known problem which the regulatory bodies aim to reduce during the treatment phase before its release into the environment, or re-using the treated sludge.

To address these concerns, the scientific community has over the years investigated EDCs in order to develop a collective understanding about their fate and behaviour in the environment as well as their toxicological effects to organisms at different trophic levels. The partial or complete degradation of several organic EDCs in WWTPs using bacterial activity and activated carbon has been reported.[78–82] Under various treatment conditions, bacteria can break down EDCs, thereby reducing the ED (endocrine disruptor) activity in the wastewater. Matsui and co-workers[83] reported a significant decrease in estrogen activity during the bacterial denitrification stage in a WWTP and similar findings were confirmed by Andersen *et al.*[84] The results suggest a pivotal role played by bacteria in ED activity reduction in WWTP.

Other studies[81,85] have also shown that increased residential time in bacterial treatment, for example nitrifying bacteria, often result in improving the reduction of ED activity in the wastewater. For instance, a recent review by Liu *et al.*[86] on EDC removal mechanisms in wastewater concluded that the bacterial activity was comparatively more efficient at reducing their activity than physical and chemical phases—although the latter two approaches still play a significant role along the treatment chain.

3. Antibacterial toxicity of ENMs

Any chemical substance that inhibits or terminates the growth of a bacterial cell, population or community is regarded as possessing antibacterial activity, and generically referred to as an antibacterial agent.[55] Such substances can either be natural or synthetic materials. More than 800 forms of proteins and peptides in the plant and animal kingdom exhibit antibacterial performance.[87] In the modern era, numerous synthetic materials have been developed or are in the research and development phase in the health sector for domestic (soaps, detergents) or medical (antibiotics, sterilants) applications to fight disease-causing bacteria. Antibacterial agents can inhibit growth (bacteriostatic), damage cells (bacteriolytic) or kill cells (bacteriocidal), collectively called antibacterial activity, and generally measured through the determination of the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC).[36,55]

There are various modes of antibacterial toxicity, including attacks on the cell wall, cytoplasmic membrane, protein synthesis, and nucleic acids synthesis.[55] The preceding steps towards antibacterial toxicity for each mode of mechanism on these target sites are highly variable, as they could be based on a variety of chemical or physical pathways. Lately the antibacterial toxicity of ENMs has attracted increasing scientific investigation with TiO_2 and AgNPs being the most studied.[36] Antibacterial activity of ENMs is induced following toxicity routes discussed earlier and Klaine and co-workers[36] have also give a detailed discussion on this issue.

Other studies have confirmed the antibacterial activity of ENMs through the disruption of the cell membrane,[15,88,89] which often occurs through the alteration of permeability and fluidity caused by the generation of ROS. The membrane-oxidising ROS can also affect energy conversion pathways, for example through oxidation of membrane components involved in energy pathway and also disrupting membrane bound electron carriers thereby affecting the transport of electrons in the energy pathway.[90–92] Additionally, ROS can disrupt the integrity of proteins as well as their synthesis through chemical oxidative interactions and physical electrostatic interactions.[93,94] Both the primary modes of action as well as secondary modes are highlighted to illustrate the antibacterial toxicity of the ENMs under review.

In this section, toxicological effects of several ENMs to bacteria are presented. Over the last few years, increasing numbers of publications have appeared highlighting the interactions of ENMs with microbial communities (Table 3). In this section, only few examples for each of the ENMs selected are discussed, namely; nC_{60}, CNTs, $nTiO_2$, AgNPs, and nZnO.

3.1. Silver nanoparticles

The antibacterial properties of silver and its compounds are well known and have been beneficially manipulated for centuries. AgNPs are known to exhibit more effective antibacterial properties than bulk silver, which has led for the former to receive increasing attention in the scientific and technology areas,[95–101] for example in fabric sterilisation, antibacterial wound dressing and water purification. The size effect of AgNPs has been shown to improve fabric sterilization against bacteria and fungi[98,102–104] and it is these nano-size driven beneficial effects that are driving the huge interest in AgNPs.

AgNPs are toxic to a variety of bacteria including several antibiotic resistant strains such as *Streptococcus* sp., *Pseudomonas* sp., *Streptococcus* sp. and others.[105–107] Therefore, in the field of medical biotechnology there exists a wide knowledge about the use of AgNPs toxicity to combat antibiotic resistance in pathenogenic bacteria, wound infectious bacterial strains, and for destroying viruses such as human immunodeficiency virus (HIV).[108,109]

In a laboratory scale bioreactor the AgNPs were found to reduce the activity of nitrifying bacteria by up to 41.4%, and were more toxic than Ag^+ where the latter reduced the bacteria activity by 13.5%.[110] The antimicrobial property of AgNPs has motivated the increased interest towards understanding its mode of toxicity, with many studies on AgNP microbial toxicity and underlying modes. The microbial toxicity of AgNPs is dependent on physicochemical properties such as size and shape. Smaller sized particles ($\leq$10 nm) were highly toxic[38,96,100,111–114] (because the small size increases the generation of Ag^+[38]). Triangular-shaped nanoparticles were more toxic than spherical and rod-shaped nanoparticles because they had a higher density of atoms per unit area on the edges.[115]

Shrivastava *et al.*[116] postulated that the major mechanism through which silver nanoparticles manifest antibacterial properties was by anchoring to and penetrating the bacterial cell membrane (Fig. 1A–1C). Another mode of AgNPs bacterial toxicity is through the induction of oxidative stress[98,112] (Fig. 1D). Hwang *et al.*[88] observed that Ag^+ induced the same effect in bioluminescent bacteria sensitive to membrane protein damage and slightly less effect in a strain sensitive to superoxides compared to AgNPs. The findings suggested that AgNPs produced Ag^+ that moves inside the cells resulting in the generation of ROS by redox reactions with oxygen. Similarly, the bacterial activity of activated carbon fiber supported silver has been attributed to the synergistic action of silver ions, superoxides and hydrogen peroxide.[117]

Table 3 Summary of the antibacterial effects of carbon- and metal-based engineered nanomaterials[a]

ENP type	Bacteria type	Physicochemical properties studied/reported	Characterization techniques	Suspension media/preparation method	Main findings (values in mg L^{-1})	Reference
$nTiO_2$	*Vibrio fischeri*	Size = 20–70 nm	No characterization techniques reported. Most likely size value as per manufacturer specification	Milli-Q water, sonication for 30 min	No impairment on growth reported. EC_{50} (nano): >20 000; EC_{20} (nano): >20 000; NOEC: >20 000; MIC: >20 000	196
$nTiO_2$	*Escherichia coli*	Size: 66 nm, 950 nm, 44 mm No coating. No impurities specified	DLS	Rigorous shaking	Growth inhibition. No inhibition at 100 mg L^{-1} 15% inhibition at 500 mg L^{-1}	41
$nTiO_2$	*Escherichia coli*	Size: 79 nm (Aeroxide P25; 80% anatase, 20% rutile)	DLS	Ultrasound in ultrapure water and sonicated	25% of bacteria survival at 1200 μM (≈ 100 ppm), and no effect at 140 μM (≈ 11 ppm) was observed	136
THF/nC_{60}	*Escherichia coli*	Avg. diameter of THF/nC_{60} = 64 nm	DLS	Solvent (THF)	Showed high toxicity regardless of light presence (at 140 μM (≈ 11 ppm)) almost 100% mortality of bacteria	136
nC_{60} (aqu/nC_{60})	*Escherichia coli*	Avg. diameter of aqu/nC_{60} = 84 nm	DLS	Sonication in ultrapure water	Limited antibacterial activity regardless of light exposure at 140 μM (≈ 11 ppm) (photochemically inert and unharmful to bacteria)	136
PVP/C_{60}	*Escherichia coli*	Avg. diameter of PVP/C_{60} = 4.4 nm	DLS	PVP used in encapsulating C60 molecules		136
$nC_{60}(OH)_{24}$	*Escherichia coli*	Avg. diameter of Fullerol = 122 nm (hydroxylated C_{60})	DLS		Limited antibacterial activity regardless of light exposure at 140 μM (≈ 11 ppm)	136
aq/nC_{60}	*Bacillus subtilis*	Diameter ≈ 30–300 nm	DLS, TEM		MIC = 0.5 ± 0.13 mg L^{-1} (smaller particles (amorphous) were more antibacterial compared to larger ones (crystalline))	128
THF/nC_{60}	*Bacillus subtilis*	Diameter ≈ 50–150 nm	DLS, TEM		MIC = 0.09 ± 0.01 mg L^{-1} (smaller particles (amorphous) were more antibacterial compared to larger ones (crystalline))	128
PVP/ nC_{60}	*Bacillus subtilis*	Diameter ≈ 10–25 nm	TEM		MIC = 0.95 ± 0.35 mg L^{-1}	128
son/ $nC_{60}(OH)_{24}$	*Bacillus subtilis*	Diameter ≈ 10–25 nm	TEM		MIC = 0.7 ± 0.3 mg L^{-1}	128
nC_{60}	*Bacillus subtilis*	Size; 3–11 384 (control to highest treatment). Functionalized nC_{60} also studied	DLS and electron microscope	Solvent (THF)	MIC = 1.5–3 mg L^{-1}; MBC = 2–4 mg L^{-1}	126
nC_{60}	*Escherichia coli*	Size; 3–11 384 (control to highest treatment) Functionalized nC_{60} also studied	DLS and electron microscope	Solvent (THF)	MIC = 0.5–1 mg L^{-1}; MBC = 1.5–3 mg L^{-1}	126

Table 3 (*Contd.*)

ENP type	Bacteria type	Physicochemical properties studied/reported	Characterization techniques	Suspension media/preparation method	Main findings (values in mg L^{-1})	Reference
nC_{60}	*Bacillus subtilis*	Size: not specified Conc.: 0.04, 0.4, 4. 0.01% carbon impurities	DLS	Solvent (THF) Stirring in milliQ water for 24 h. Aggregates: 25–500 nm	Inhibition of respiration at 4 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). No inhibition at 0.4 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). No inhibition $\leq$ 2.5 mg L^{-1}(anaerobic & aerobic conditions; Luri broth (LB) media used).	11
nC_{60}	*Bacillus subtilis*	Size: not specified Conc.: Not specified 0.01% impurities -unspecified	DLS	Solvent (THF) Stirring in milliQ water for 24 h. Aggregates: 25–500 nm	No growth above 0.4 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). Growth at 0.04 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). Growth $\leq$ 2.5 mg L^{-1} (anaerobic & aerobic conditions; Luri broth (LB) media used).	11
nC_{60}	*Escherichia coli*	Size: not specified Conc.: 0.04, 0.4, 4. 0.01% impurities -unspecified	DLS	Solvent (THF) Stirring in milliQ water for 24 h. Aggregates: 25–500 nm	No growth above 0.4 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). Growth at 0.04 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). Growth $\leq$ 2.5 mg L^{-1} (anaerobic & aerobic conditions; Luri broth (LB) media used).	11
nC_{60}	*Escherichia coli*	Size: not specified Conc.: 0.04, 0.4, 4. 0.01% impurities unspecified	0.01% impurities unspecified	Solvent (THF) Stirring in milliQ water for 24 h. Aggregates: 25–500 nm	Inhibition of respiration at 4 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used) No inhibition at 0.4 mg L^{-1} (anaerobic & aerobic conditions; minimal Davis (MD) media used). No inhibition $\leq$ 2.5 mg L^{-1}(anaerobic & aerobic conditions; Luri broth (LB) media used).	11
nC_{60}	*Bacillus subtilis* (Gram-Positive)	Conc.: 11 mg L^{-1} C_{60}. Size: mean diameter; 95 nm	DLS	Stirred overnight in 4 L of solvent nitrogen-sparge and THF	Responded to a low dose of 0.01 mg L^{-1} by significantly increasing levels of iso- and anteiso-branched fatty acids (from 5.8 to 31.5% and 12.9 to 32.3% of total fatty acids, respectively). Growth-inhibition conc. of 0.75 mg L^{-1} (MIC between 0.5 to 0.75 mg L^{-1})	198
nC_{60}	*Pseudomonas putida* (Gram-negative)	Conc.: 11 mg L^{-1} C_{60}. Size: mean diameter; 95 nm	DLS	Stirred overnight in 4 L of solvent nitrogen-sparge and THF	Decreased its levels of unsaturated fatty acids and increased the proportions of cyclopropane fatty acids in presence of nC_{60}, possibly to protect the bacterial membrane from oxidative stress (effects observed at 0.01 mg L^{-1}). Growth-inhibition at 0.5 mg L^{-1} of nC_{60}.	198

Table 3 (*Contd.*)

ENP type	Bacteria type	Physicochemical properties studied/reported	Characterization techniques	Suspension media/preparation method	Main findings (values in mg L^{-1})	Reference
nZnO	*Vibrio fischeri*	Size = 50–70 nm	No characterization techniques reported. Most likely size value as per manufacturer specification	Sonication for 30 min in deionised water and stored in dark at 4 °C. Before toxicity testing	LC_{50} (nano): 3.2 LC_{20} (nano): 2.45 NOEC: 0.5\67–97% bioavailable (average 83%)	196
nZnO	*Bacillus subtilis*	Size: 67 nm, 820 nm, 60 mm; No coating. No impurities specified	DLS	Rigorous shaking	Growth inhibition (90% inhibition at 10 mg L^{-1})	41
$nZnO_2$	*Escherichia coli*	Size: 67 nm, 820 nm, 60 mm; No coating. No impurities specified	DLS	Rigorous shaking	Growth inhibition. 14% inhibition at 10 mg L^{-1}	41
nZnO	*Staphylococcus aureus*	50–70 nm particle diameter. More than 99% pure	Tryptic soy broth (TSB)	Shaking at 200 r.p.m.	EC_{50} = 5 mM; MIC = 15 mM or 1.2 mg mL^{-1}	169
nZnO	*Staphylococcus epidermidis*	50–70 nm particle diameter. More than 99% pure	TSB	Shaking at 200 r.p.m.	EC_{50} = 5 mM; MIC = 15 mM or 1.2 mg mL^{-1}	169
nZnO	*Streptococcus pyogenes*	50–70 nm particle diameter. More than 99% pure	TSB	Shaking at 200 r.p.m.	EC_{50} = 5 mM; MIC = 15 mM or 1.2 mg mL^{-1}	169
nZnO	*Enterococcus faecalis*	50–70 nm particle diameter. More than 99% pure	TSB	Shaking at 200 r.p.m.	EC_{50} = 5 mM; MIC = 15 mM or 1.2 mg mL^{-1}	169
nZnO	*Bacillus subtilis*	50–70 nm particle diameter. More than 99% pure	Luria–Bertani (LB)	Shaking at 200 r.p.m.	EC_{50} = 5 mM; MIC = 15 mM or 1.2 mg mL^{-1}	169
nZnO	*Escherichia coli*	50–70 nm particle diameter. More than 99% pure	LB	Shaking at 200 r.p.m.	EC_{50} = 5 mM; MIC = 15 mM or 1.2 mg mL^{-1}	169
nZnO	*Escherichia coli* (Gram-negative)	40–350 nm particles	Luria–Bertani (LB)	Cultured in minimal essential medium (MEM) with 10% fetal bovine serum (FBS)	Membrane-damage mechanism of antibacterial action in favour of an ROS model	183
nZnO	*Staphylococcus aureus* (Gram-positive)	1.2 μm particles	Brain heart infusion (BHI)	Cultured in minimal essential medium (MEM) with 10% fetal bovine serum (FBS)		183
nZnO	*Escherichia coli* (Gram-negative)	10–30 nm particles	RPMI 1640 medium	Washed in distilled water and centrifuged at 3000 rpm	Induced apoptosis MIC = 500 μg mL^{-1}	180
nZnO	*Pseudomonas aeruginosa* (Gram-negative)	10–30 nm particles	RPMI 1640 medium	Washed in distilled water and centrifuged at 3000 rpm	MIC = 500 μg mL^{-1} Induced apoptosis	180
nZnO	*Staphylococcus aureus* (Gram-positive)	10–30 nm particles	RPMI 1640 medium	Washed in distilled water and centrifuged at 3000 rpm	MIC = 125 μg mL^{-1} Induced apoptosis	180
AgNP	*P. aeruginosa*, *V. cholera*, *E. coli*, *S. typhus*	16; 0–100 μg mL^{-1}	TEM, EDS, HAADF STEM	H_2O suspended	30 min bacterial growth; growth inhibition	96
AgNP	*Escherichia coli*	12.3 (average); 10–100 μg cm^{-3}. 158 m^2 g^{-1} (SSA)	TEM, Micromeritics Flowsorb II	dH_2O, mild ultrasonication	24 h bacterial growth; 70 and 100% bacterial growth inhibition at 20 and 50–60 μg cm^{-3}	114

Table 3 (*Contd.*)

ENP type	Bacteria type	Physicochemical properties studied/reported	Characterization techniques	Suspension media/preparation method	Main findings (values in mg L^{-1})	Reference
AgNP	*Escherichia coli*	39 (mean), 1–100 μg 100 mL^{-1}	ICP MS, ICP ES. EFTEM, UV-Vis spectroscopy, image tool software	Nutrient broth, ultrasonication	24 h bacterial growth; (1) truncated triangular particles ≈ EC_{100} = 1 μg cm^{-3} (2) spherical particles: EC_{100} = 50–100 μg mL^{-1}	115
AgNP	*Escherichia coli*	<10, 4 (average)	TEM, XRD and UV-Vis spectroscopy	Luria–Bertani medium	Bacterial growth; EC_{100} = 22.64 and 28.3 μg mL^{-1}	184
AgNP	*Escherichia coli* and autotrophic bacteria	14 (average); 1 mg L^{-1}	UV-Vis spectroscopy, STEM	8.3 mM NH_4NO_3, pH 7.5	Bacterial growth; 86% respiration reduction. 55% *E. coli* growth reduction	112
AgNP	*Escherichia coli*	20 (average); 0–40 μg mL^{-1}	TEM, UV-Vis spectroscopy, image tool software	Luria–Bertani medium	24 h bacterial growth; NOEC = <30 μg mL^{-1}, LOEC = 40 μg mL^{-1}	106
AgNP	*Nitrosomonas*, *Nitrobacter* and *Nitrospira* sp.	21 (average); 1 mg L^{-1}	TEM	Modified Lud-zack-Ettinger activated sludge	12 h nitrifying activity inhibition; 44% nitrification reduction	110
AgNP	Nitrifying bacteria	9–21 (average); 0.05–1 mg L^{-1}	TEM, UV-Vis spectroscopy	8.3 mM NH_4NO_3, pH 7.5	Bacterial growth; Growth inhibition, EC_{50} = 0.14 mg L^{-1}	112
AgNP	*Escherichia coli* and *Staphylococcus aureus*	13.4 (average); 0.2–33 nM. Zeta potential: slightly negative (0–1)	TEM, HRTEM	Muller Hinton agar	24 h bacterial growth; Growth inhibition, (*E. coli*) LOEC = 3.3–6.6 nM. (*S. aureus*) LOEC = 33 nM	98
AgNP	*E. coli*, *S. aureus*, *S. typhus*	5–35 μg mL^{-1}	TEM	Milli-Q deionized water	24 h bacterial growth; $EC_{60,90,100}$ = 5, 10, 25 μg mL^{-1} (*E. coli*); $EC_{70-75,100}$ = 10, ≥25 μg mL^{-1} (*S. typhus*), no effect observed for *S. aureus*	116
AgNP	*Escherichia coli*	6.7; 1–50 (Ag/SiO_2; C = 1 mg L^{-1})	STEM, HRTEM, EDXS	LB broth, DI water	330 min bacterial growth	38
CNTs	*Escherichia coli*	0.57–1.2; 1–50 μg mL^{-1}	TEM	Not reported	60 min and 30–60 min, viability loss; (1) 73.1, 79.9 and 87.6% cell viability loss in 30, 60 and 120 min respectively	139
CNTs	*Escherichia coli*	0.9 (average). (SWNT; length not reported)	TEM, SEM	Aqueous solution	60 min cell viability; 79.9% inhibition	139
CNTs	*Escherichia coli*	0.9; 30: SWNT; MWNT; 5 μg mL^{-1}. (2; 70 μm: SWNT; MWNT length)	TEM	Saline solution	60 min cell viability; 80% (SWNT) and 24% (MWNT) cell inhibition	140
CNTs	*E. coli*, *P. aeruginosa*, *B. subtilis*, *S. epidermidis*	1.2; 17.4: SWNT; MWNT. (17.8; 77: SWNT; MWNT length)	TEM, SEM, thermo-gravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS)	CBN-coated filter in 0.154 M isotonic solution	60 min direct contact cellular toxicity; Significant toxicity (fluorescence-based) induction for all species on contact with MWNT and SWNT	141

Table 3 (*Contd.*)

ENP type	Bacteria type	Physicochemical properties studied/reported	Characterization techniques	Suspension media/preparation method	Main findings (values in mg L^{-1})	Reference
CNTs	*Escherichia coli*	1.2 (average); 0.3–0.8 mg cm^{-2}. 407 m^2 g^{-1} (SSA). 10–20 μm (length)	SEM	SWNT filter	20 min cell viability; 79% cell inactivation (fluorescence based)	197
CNTs	*S. typhimurium*	1–1.5; <1–5 μm (length)	SEM (EDX), TEM	DI water	60 min cell viability; Significant antimicrobial activity at all concentrations	145
CNTs	*S. typhimurium*, *S. aureus*, *B. subtilis*	1.5, 15–30 (SWNTs, MWNTs); 10, 1–5 μm (SWNTs, MWNTs; length); 100–500 μg mL^{-1}	SEM	DI water and 0.9% NaCl	60 min cell viability and bacterial growth; SWNTs induce high antimicrobial activity, MWNTs show no antimicrobial activity up to 500 μg mL^{-1}	147

[a] DLS: dynamic light scattering device; MIC: minimal inhibitory concentration (MIC); MBC: minimal bactericidal concentration; PVP: poly(*N*-vinylpyrrolidone); SSA = specific surface area, NB = nutrient broth.

Results on the effect of coating and functionalization on the antibacterial properties of AgNPs are controversial and conflicting. For example, one study reported that AgNPs coated with sodium dodecyl sulfate (Ag-S) had no antibacterial activity,[118] while Kvitek and colleagues[119] found Ag-SDS to exhibit the most effective antibacterial activity of all nano-Ag tested. Furthermore, abiotic factors such as pH, concentration, and NOM have also been shown to influence the antibacterial properties of AgNPs.[120] NOM was observed to mitigate the toxicity of nanoparticles due to their sorption on the AgNPs surfaces, preventing the interaction of nanoparticles with the bacteria.[120]

To date, only limited studies have illustrated the potential effect of AgNPs on useful bacteria in WWTPs, for example nitrifying bacteria.[112,121,122] Autotrophic nitrifying bacteria that are essential for the nitrification process critical in biological nutrient removal in wastewater are susceptible to inhibition (*e.g.* inhibited respiration by 86 ± 3%) by Ag NPs.[121] Such results indicate that the accumulation of AgNPs may cause detrimental effects on the essential microbial ecology of wastewater treatment systems. This implies that AgNPs toxicity towards bacteria may potentially in future require stringent regulations to protect WWTP systems integrity from the effects of AgNPs.

3.2. Carbon fullerenes (C_{60})

Earlier studies on C_{60} reported growth inhibition and bactericidal effects, mainly on pathenogenic bacteria, thereby promising effective antimicrobial properties against infectious bacterial strains.[123–125] In later years, the increased production of various forms of fullerene derivatives was driven by potential biomedical applications. Antibacterial investigations on fullerenes have also focused on their effects on environmental microbial ecology in water and in soil compartments.[11,126–129] These studies indicated various levels of inhibition and bactericidal properties on bacterial populations in water and soil, thereby raising concerns and uncertainties about the environmental impacts resulting from fullerene nanowastes disposal.

Microbial diversity and activity in WWTPs and receiving waters face increasing level of risks from carbon based nanomaterials due to observed reduction in cell viability and cell membrane integrity on bacteria exposed to CNTs.[130] Some research links the antimicrobial and antiviral toxicity of fullerenes to the production of pro-oxidant ionic forms and oxidative stress that can result to genetic and protein effects.[131–136] However, the results of other researchers suggest that such toxicity is not oxidative stress mediated, or that oxidative stress is an insignificant toxicity route.[137,139] Brunet *et al.* argued that tests investigating *in vitro* effects of ENMs and the production of ROS should be performed using water with the same chemistry water to eliminate exposure media influence due its potential of lessening or masking the oxidative toxicity significance.[136] The antimicrobial activity of fullerenes is however an indisputable and well reported issue supported by increasing scientific evidence.[130–138]

3.3. Carbon nanotubes (CNTs)

The antimicrobial property of single-walled carbon nanotubes (SWCNT) was reported by Kang and co-workers.[139] Their study showed a loss of cell viability and damage to bacterial exposed to

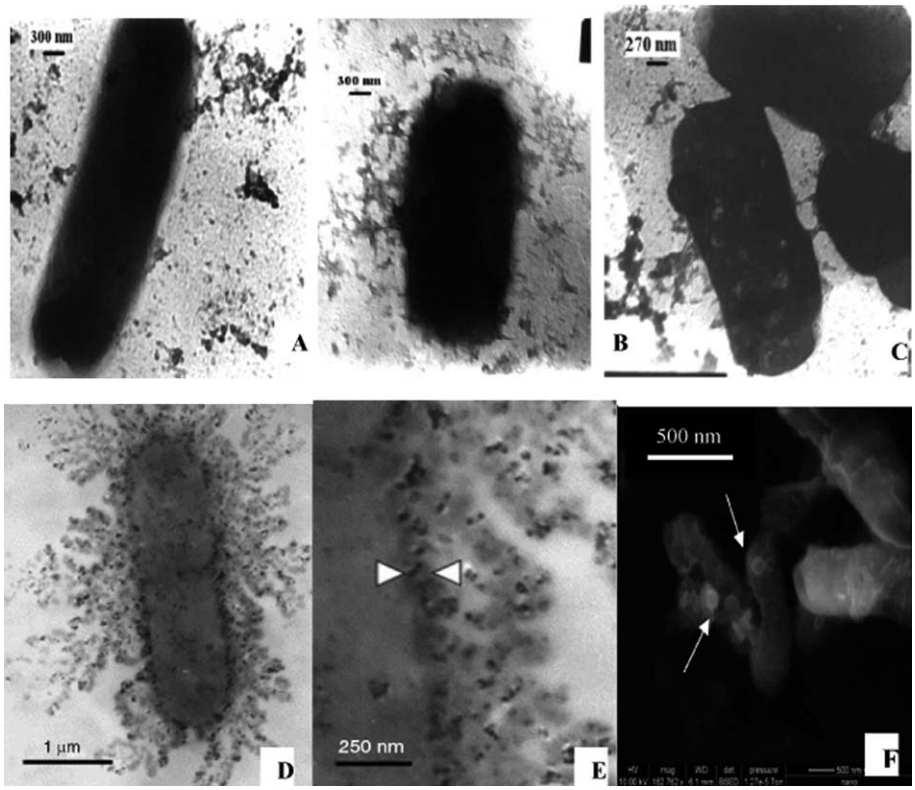

Fig. 1 Illustrative evolution on the interaction between gram-negative bacteria (*Staphylococcus aureus*) and AgNPs using transmission electron microphotographs. In the initial stages of interaction (A), clusters of NPs were found to anchor onto the bacterial cell wall, possibly at sites that are rich in negatively charged functional groups. After sometime (B) the nanoparticles manage to enter the bacterial cell, and in other cases (C) the nanoparticles were observed to anchor the cell at several sites and make perforations in the membrane with a possibility of resulting in cell lysis.[116] Another example is the transmission electron microphotograph of *Escherichia coli* with AgNPs in liquid Luria broth medium which caused extensive cell membrane damage (D), and the enlarged view of the membrane of this cell (E),[114] and finally the spherical or hexagonal types of AgNPs attached to the microbial cells of nitrifying bacteria, probably causing cell wall pitting (F).[121]

SWCNT particularly due to morphological change (Fig. 2). Follow up studies by Kang and co-workers[140–142] further probed carbon-based nanomaterial antibacterial activity and showed that exposure increases the expression of stress related genes, causes cell membrane disruption, and increases cytotoxicity. The CNTs toxicity is influenced by size diameter and SWCNT are relatively more toxic to bacteria than MWNTs and fullerenes.[142]

Brady-Estevez *et al.*[143] reported SWCNT bacterial toxicity where a SWCNT impregnated filter was used to remove microbial pathogens in water. The results indicated an increased number of dead cells and reduced metabolic bacterial activity in water passed through the filter, further providing evidence of antibacterial activity of CNTs. Later Brady-Estevez and co-workers[144] showed the antiviral properties of CNTs where the MWCNTs were found to be more antiviral than SWCNT. With regard to the underlying physicochemical parameters influencing bacterial toxicity the size and length of CNTs were found to significantly influence toxicity activity. For instance, smaller and longer CNTs were found to be more antibacterial possibly due to their high degree of dispersion in solution.[140,141,145]

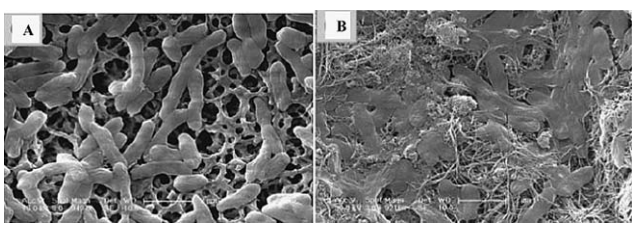

Fig. 2 Scanning electron microscope (SEM) images of *Escherichia coli* illustrating the antibacterial property of SWCNT after interacting for 60 min with: (A) cells incubated without SWCNT, and (B) cells incubated with SWCNT.[139]

Most importantly there is a growing consensus that membrane integrity disruption through physical and electrical interaction may account for the release of intracellular contents which underlines the mode of bacterial cytotoxicity.[36,93,94,146] Although no single factor can be highlighted as the most important driving factor, several parameters such as size, length, surface functional group, aggregation state and dispersion state are among those which have been correlated to bacterial cytotoxicity. Evidence of bacterial oxidative stress response gene expression links oxidative stress as one of the underlying toxicity mechanisms,[140] a possibility further strengthened by recent studies reporting the oxidative cellular membrane integrity disruption by the CNTs.[145,147]

3.4. Titanium dioxide (TiO_2)

The bactericidal effects of TiO_2 have been known and utilised as early as 1985.[148–151] The discovery of bacterial toxicity of TiO_2

drove interest in sterilisation against bacteria, fungi and viruses as well as in killing cancerous cells. Sunada *et al.*[152] and Blake *et al.*[153] described the generation of free radicals as the underlying route for $nTiO_2$ antimicrobial toxicity, while Maness *et al.*[154] reported an increased lipid peroxidation which resulted in inactivation and viability loss of bacterial cells.

Sunada *et al.*[155] later confirmed previous findings[152] by suggesting that $nTiO_2$ microbial toxicity followed a two step mode: the oxidative destabilisation of the outer cellular membrane through lipid peroxidation and thereafter an attack of the cytoplasmic membrane by free radicals. Recently Lin *et al.*,[156] suggested the $nTiO_2$ antibacterial properties were due to oxidative stress. Other recent studies have also highlighted oxidative toxicity, cellular membrane integrity destabilisation and generation of hydroxyl radicals as the routes through which $nTiO_2$ affects bacterial activity and growth rates,[136,157–160] and some of these mechanisms are illustrated in Fig. 3.

As in other cases of ENMs toxicity, $nTiO_2$ toxicity is influenced by physical properties like size and crystallinity.[157,158] $nTiO_2$ showed an ability to alter the bacterial nitrogen-fixing activity of *Anabaena variabilis* through a dose dependant induction (concentration and time) increase in both the occurrence and intracellular levels of the nitrogen-rich cyanophycin grana proteins (CGPs),[159] which also act as detoxifying agents against protein destabilisation.[162] The study demonstrated that nitrogen-fixing activity may be hampered by the release of $nTiO_2$ into the aquatic environments with consequential disruptions of important biogeochemical processes, such as nutrient cycling. Notably, in most studies highlighted in this review, the reported toxicity was in parts per thousand concentrations which suggest low $nTiO_2$ environmental risk because such concentrations are unlikely. However, a lack of chronic and morbidity data limits our ability to make generalizations.

3.5. Zinc oxide nanoparticles (ZnO)

By the late 1990s, the protective effect of bulk ZnO against intestinal bacterial infections was known.[163,164] The growth reduction in bacterial colony of *Escherichia coli*,[165–168] *Staphylococcus* sp., *Bacillus* sp., *Streptococcus* s. *Staphylococcus agalactiae*, *Staphylococcus aureus* was later confirmed after exposure to nZnO.[167,169,170] nZnO has also been shown to be highly antibacterial in soil colonies, so concerns on the potential impacts of nZnO to both aquatic and terrestrial populations have been raised.[170,171] This is because the biosolids from the WWTPs are largely used for agricultural applications which may result in long-term adverse effects, particularly to essential microbial soil populations.

Although the nZnO or other ENMs in the above mentioned studies were synthesized, prepared and exposed at various concentrations—the increase of antimicrobial activity for the nZnO related nanoscale properties are unknown. Further investigations into the mechanistic toxicity of nZnO reveal that the toxicity was highly influenced by particle size and concentration[50,169,170,172] while the crystalline structure and particle morphology were of lesser importance.[173]

Apperlot *et al.*[174] argued that the antibacterial activity of nZnO was due to the generation of free radicals partly as a function of ENM size in suspension. Thus, current studies suggest that nZnO affects bacterial cell viability and integrity by

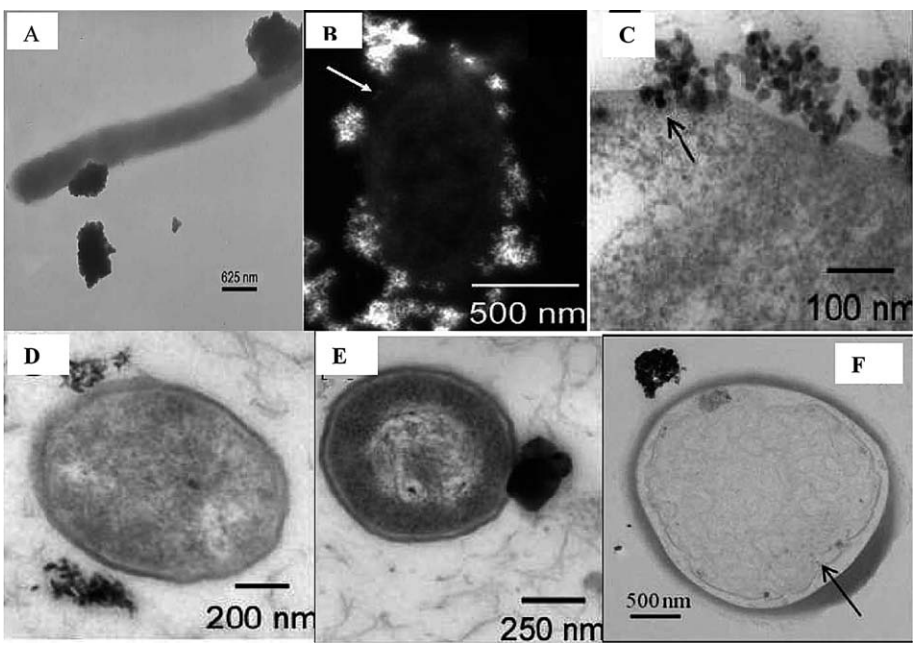

Fig. 3 Images of $nTiO_2$ interactions with different bacteria species: (A) TEM image revealing the attachment of nanoparticles to the surface of *Pseudomonas fluorescens*;[50] (B) SEM microscopy of *E. coli* MG1655 exposed to 50 mg L^{-1} of $nTiO_2$ sized 12 nm for 24 h[158] where the arrow depicts an electron-dense granule located in bacterial periplasm or of the cytoplasm of the bacterium; (C, D, E) TEM image showing the adsorption of nanoparticle in *E. coli* MG1655 of $nTiO_2$ sized 12 nm, 9 nm, 140 nm, respectively for 24 h;[158] (F) TEM image showing the adsorption of nanoparticle in *E. coli* MG1655 of $nTiO_2$ sized 12 nm for 24 h;[158] and the disruption of internal plasma membranes in heterocyst cells (see arrow) of *Anabaena variabilis* after exposure to 50 mg L^{-1} of $nTiO_2$[161]

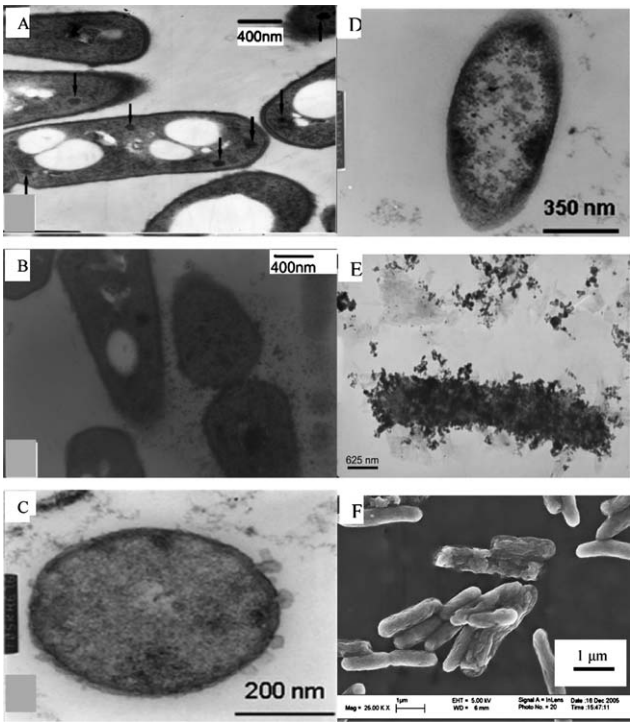

Fig. 4 (A) TEM image of *Streptococcus agalactiae* cells penetrated by nZnO, and initiation of the damage process of the cell membrane (arrows show nZnO in cells);[170] (B) TEM image depicting advanced damage of *Streptococcus agalactiae* cells;[170] (C, D) TEM images showing physical interaction of nZnO and lipid membranes of *Escherichia coli* resulting to the induction of pyridine leakage through the vesicle membrane;[172] (E) TEM images of nZnO attached to the surface of *Pseudomonus fluorescens*;[50] (F) SEM images of *Escherichia coli* showing cell membranes damage owing to interactions with 0.2 g L^{-1} ZnO nanofluids for 5 h (no cell penetration due to large agglomerates >200 nm).[176]

increasing membrane permeability and membrane disorganisation. The reduction or loss of cell viability results in reduced cell count number and colony population, which is driven by growth and multiplication inhibition. Membrane stability disruption is also due to physical interaction (nano size based electrostatic field) effects where nZnO causes membrane lipid peroxidation and loss of membrane integrity. Several studies have shown that like most other metal-based ENMs, nZnO antibacterial toxicity is also due to oxidative stress[168,170,175,176] (Fig. 4). Additionally, the generation of Zn^{2+} by nZnO when in solution is a driver for the observed antibacterial toxicity[174,177] (see Fig. 4).

4. Mechanisms of toxicity and influencing factors

Following a brief review of antibacterial activity of selected ENMs, it is important to provide a further detailed argument on mechanisms for ENMs antibacterial toxicity, and the physicochemical and environmental factors influencing such activity. In the absence of antibacterial toxicity assessments for all available ENMs, currently common toxicity routes and influencing factors are outlined below to highlight variables which could possibly be prioritised for the risk assessment of ENMs on bacterial populations. Although risk assessment can not be done on a "one size fits all" basis, we aim to discuss common antibacterial mechanisms and influencing variables in order to lay a platform for the development of risk assessment of ENMs on bacteria.

4.1. Membrane integrity disruption

Many reports suggest that the disruption of bacterial cellular membrane is one of the causes of antibacterial activity of ENMs.[15,55,88,89,116,140–142,146,155] Membrane disruption leads to a reduced ability to control the movement of substances in and out of a bacterial cell, thereby causing homeostatic imbalance, which leads to cellular metabolic disturbance and even death.[116] Membrane disruption arises in two ways: (i) strong electrostatic interaction between a negatively charged cell membrane and positively charged metal ENM, which due to their small particle size have a high surface charge. During such an interaction, metal ions released by ENMs can rupture the cell wall leading to the denaturation of membrane protein components and even cell death[146] (ii) the interaction of the ENM and the bacterial membrane can cause oxidative stress on the membrane, mediated by the generation of ROS.[88,116]

The composition of the bacterial membrane is a possible key to antibacterial activity, with the antibacterial sensitivity of gram negative bacteria being less than that of the gram positive bacteria towards ENMs.[124,178–180] The cell membrane of gram negative bacteria is multilayered, predominantly made up of tightly packed lipopolysaccharide, phospholipids and protein molecules, with an underlying thin peptidoglycan layer, which provide an effective resistive barrier against nanoparticles.[178,181,182] The cell wall of gram-positive bacteria is however mainly composed of peptidoglycan but is several layers thick.[55] This composition in gram-negative bacteria is thought to provide a more effective protective barrier than the gram-positive membranes.

Other studies have found gram-positive bacteria to be more resistant to antibacterial effect than their gram-negative counterparts.[50,183] Shrivastava *et al.* argued that the membrane of gram-negative bacteria contain a relatively high component of negatively charged components such as lipopolysaccharides, which attract the positively charged ENMs.[116] Therefore the same protective membrane constituent of gram negative makes them more vulnerable to electrostatic interaction than gram-negative species. Although gram-positive bacteria do not possess the same protective constituents in their cell membrane as gram negative species, their cell membranes are thicker, which could provide the protective layer.[50,183] Although gram negative membranes possess protective lipids and polysaccharides, these are not strongly linked and are not rigid.[116]

On the other hand, Huang *et al.*[170] have shown nZnO to be similarly bactericidal to both gram-negative *S. agalactiae*, and gram-positive *S. aureus.* Based on such conflicting information, we argue that the issue of bacteria resistance/sensitivity is not simply a function of membrane composition (gram ±), but also the physicochemical state and type of ENM, inter-species differences as well as the test conditions. Therefore, a collective understanding of such variables, especially the ENMs and media characteristics as well as species membrane composition would offer valuable insights into the possible underlying antibacterial mechanisms. We also recommend detailed reporting of such variables for ENMs antibacterial studies in order to aid the

making of scientifically sound assumptions in risk assessment, since testing of all materials is impossible.

4.2. Reactive oxygen species

After the ENMs have penetrated the cell membrane and are inside a cell, they can promote the generation of reactive species which cause peroxidation of various organelle constituents.[98,112,117] At this stage both the gram positive and gram negative bacteria would be similarly susceptible since the physical barrier of the membrane is of no significance when the ENMs are already within the cell. The induction of oxidative stress within a cell could be as a result of metal ion species released by ENMs or through direct interaction of the ENM with organelles. Secondary effects such as DNA strand breakage and protein inactivation can occur, causing cellular metabolic disruption which can finally cause cell death or apoptosis.

Actually, silver ENMs within a cell have a greater affinity for sulphur or phosphorus containing sites such as DNA, at which they can initiate their oxidative attack.[115] Most of the articles reviewed in [115] did not provide the mechanism of toxicity. Therefore, it is suggested in future toxicity studies that investigators should try to elucidate the mechanism underlying the observed toxicity. A number of mechanisms may occur simultaneously or one may trigger the other,[15,36,55,88,93,94,98,102,116,128,136,138–142,155–157,174–177] however, the results are inadequate to support the drawing of definitive conclusions at this stage.

4.3. ENM size

The influence of particle size or physicochemical properties on the toxicity potential of ENMs is well known within the relatively young field of nanotoxicology.[38,96,100,111–113] Such an influence has also been highlighted earlier in this manuscript as a significant driver towards bacterial toxicity of ENMs. We therefore suggest that size parameters of ENMs be one of the priority evaluations in the development of environmental health and safety regulations as well as risk profiling. In this case, ENM specific (case-by-case) guidelines are needed because reports suggest that the size effect does not necessarily apply to all ENMs.

4.4. Initial culture population

Some studies have concluded that the resistance of a bacterial population towards an antibacterial effect of ENMs also depends on the initial stocking density, with highly dense initial cultures being more antibacterial resistant than less dense cultures.[115,183] The basis for such an effect is that a population growth rate will be immediately reduced if the ENMs interact with a less dense population, while the effect can be delayed or minimised on populations which are highly dense and which can still reproduce even if under attack from ENMs. Such information could support bacterial stocking intensity as a risk management criterion within a WWTP, in order to minimise or avoid population stability disruption and poor treatment efficiency. Ideally the stocking density should be such that, even when faced with antibacterial activity from ENMs, a fair amount of the population should still be able to reproduce in order to counteract the ENMs antibacterial effect on the viability of the population. We therefore recommend more investigations towards the influence of stocking density, and how it can be practically monitored within a WWTP, as a measure of enhancing our understanding of the system resistance towards antibacterial effects of ENMs.

4.5. Effect of natural organic matter (NOM)

Environmental factors like NOM, ionic strength or pH influence the antibacterial properties of ENMs. Li *et al.*[51] showed that NOM reduced the bioavailability and the antibacterial activity of nC60, and the sorption capability depended on soil type, even at a NOM concentration as low as 0.05 mg L^{-1}. The findings suggest that NOM may protect microbial populations from adverse effects of nC_{60} due to its high abundance in soil environment. Bradford *et al.*[184] reported that AgNPs had no effect on bacterial activity in estuarine sediments even at concentrations as high as 1000 μg L^{-1}, which is much higher than future expected values of AgNPs in the actual environment.[15] The shielding effect was attributed to elevated concentrations of the chloride ions in saline estuary water, which modified the chemistry and behaviour of AgNPs. Metal ions are generally known to form ionic complexes with chloride ions in saline water which then masks their toxicity potential.

However, whether these findings can hold in WWTP remains unclear, given the low concentrations of chloride ions in such systems. Also, the extent to which the NOM can be presumed to effectively protect the microbial communities remains an open question, given the increasing concentrations of ENMs as the nanotechnology application increases, compounded by the large diversity of NOM types. Recent findings suggest that the type of NOM source strongly determines the extent of ROS generation and adsorption of AgNPs as the humic acid (HA) differ with the source. Fabrega *et al.*[120] reported that HA could act as a physical barrier to cell–nanoparticle interactions and also as an antioxidant by reacting with ROS, mitigating short term bacterial toxicity caused by AgNPs to *Pseudomonas fluorescens*.

In summary, most of the toxicity data presented in the literature on the antibacterial effects of ENMs were obtained in relatively simple media, such as distilled water or cell culture media, which do not reflect the aquatic environment inside living organisms, nor the natural environment. Therefore, the surface chemistry, reactivity and state of dispersion achieved in the laboratory may not be relevant for assessing behaviour of ENMs in real systems and their interaction with the microbial communities. This is because actual environmental compartments generally, and particularly WWTPs, are characterized by wide variations of pH, ionic strength, ionic composition and NOM. Consequently, these factors are likely to cause widely varied aggregation states of the ENMs which may result in a large spectrum of varying antimicrobial activities and toxicities.

In addition, most toxicity data are obtained by dispersing ENMs in or on nutrient rich growth media, which are significantly different to conditions in actual environmental systems. Therefore, these aspects merit attention in future research, which should investigate the underlying factors to account for the observed variations in toxicological effects. Factors to be considered include the transformation of the physicochemical properties of ENMs as a result of the environmental factors and

the metrology used in quantifying each of the influencing factors. However, though the currently accessible data have limited environmental relevance, they are important building blocks towards understanding the behaviour and fate of ENMs in different environmental conditions Modelling can provide additional information, and significant current knowledge of the behaviour and fate of macroscale pollutants in the environment has been derived through coupling of laboratory experiments with modelling results. Such approaches also have potential for application in the case of nanoscale-based pollutants.

5. Environmental significance of antibacterial properties of ENMs

The advent of nanotechnology is characterised by increased production of consumer nanoproducts as well as industrial applications with unintended releases of ENMs into the natural and engineered environments. Therefore, in addition to the undesired potential efficiency alterations of WWTPs by ENMs, the effluent and sludge discharges from compromised WWTPs have several implications to the receiving environments. In this section, the potential aquatic and terrestrial environmental implications related to the antibacterial properties of ENMs in WWTPs are highlighted.

Changes of the chemical composition of inflow to WWTPs can alter treatment efficiency, for example by growth inhibition of certain bacteria.[185] This is a consequence of a shift in bacterial population composition or activity. For instance, the inhibition of ammonia oxidizing bacteria would result in reducing the nitrification of ammonium in wastewater thereby posing risks to the ecology and to human health.[186,187] Consequently, eutrophication and community changes in receiving water systems are among the potential adverse effects of altered WWTP bacterial dynamics. Nitrification by bacteria is a key component of global nitrogen cycling, for example by the *Nitrobacter* spp. which are active in soil and freshwater environments.[188] In the case of ENMs, AgNPs[103,189,190] are being exploited as antibacterial agents for treating water and wastewater, and compelling evidence exists that the same useful properties may equally cause unintended effects to essential bacteria such as nitrifying bacteria in the environment.[112,121,122]

Bacteria are beneficially utilised for the reduction or alteration of persistent organic contaminants such as antibiotics in WWTPs, so as to reduce harm to biota in the environments receiving WWTPs effluents. Batt *et al.*[191] reported chronic antibiotic exposure of receiving environments due to incomplete elimination of antibiotics during treatment in WWTPs. The persistence of antibacterial effects of such chemicals poses risks not only for the receiving bacterial populations but also to other biota which could have their metabolism altered though such exposures. Antibiotics have endocrine disrupting capability and currently are of global concern due to their persistence in the environment as well as their metabolism influencing action. Watkinson and colleagues[192] reported the presence of antibiotics in receiving waters, and the findings of Miao *et al.*[193] confirmed the occurrence of various antibacterial substances in the final WWTP effluents—and suggested the possibility of antibacterial risks to the surface water at the discharge points.

In the light of the above information, it is clear that contaminants from WWTPs will eventually be released into the receiving waters, and the situation is worse in efficiency compromised WWTPs. Some of those chemical contaminants are targets of biological treatment in WWTPs, so their degree of degradation depends highly on the viability of bacterial populations. We therefore argue that the antibacterial activity of ENMs in WWTPs means that some chemicals which are bacterially decomposed can escape at increased concentrations into the receiving environment. In addition ENMs may potentially be discharged from the WWTPs and pose a risk to the integrity of receiving environments due to their antibacterial activity. However, due to already discussed stability dynamics of ENMs in wastewaters and lack of quantification methods and studies in WWTPs, it is currently difficult to quantify the scale of possible direct and indirect ENMs impacts on receiving environments. Given the current data limitations, we suggest that the antibacterial properties of ENMs are not only a quality control issue in WWTPs but also have implications for environmental monitoring.

Concerning environmental monitoring of ENMs, it should be noted that in actual environmental systems, ENMs may not easily come into contact with bacteria, which is a pre-requisite for the reported toxicological effects reviewed in this article. Rather, in the engineered and natural systems, the presence of other reactive chemical and organic species may limit such interactions. On the other hand, given the high tendency of bacteria to attach in aquatic[194] and soil environments[195] rather than exist as free cells, may limit our ability to estimate the full potential impact of ENMs in the environment based on the published data.

6. Concluding remarks

The inconsistencies in the literature on the parameters discussed here (ENMs size, initial stocking density, type of bacteria, NOM, *etc.*) that influence the antibacterial effects of ENMs, paints a picture that "one size does not fit all". In the midst of such data variation, we suggest that more scenario-specific ENMs antibacterial investigations, for example using wastewater from WWTPs, will provide close to real scenario risk estimations. The data and knowledge from such studies will be useful in developing guidelines for safeguarding the integrity of microbial populations in WWTPs, those in agricultural soils which can be adversely impacted after the application of biosolids, as well as populations in effluent-receiving aquatic resources. Such findings may also support the development of environmental safety regulations.

Although the stability, fate and behaviour of ENMs in actual WWTPs are difficult to determine, some parameters which can assist with making deduction on potential ENM–bacteria interaction, such as type of bacteria and stocking density are relatively simple to measure. In addition, the concentrations of the ENMs in the WWTPs can be evaluated from the expected volumes of nanoproducts, and ENMs size parameters can be estimated using a combination of available techniques such as SEM, TEM and DLS. This information, although incomplete, would aid in risk profiling of different ENMs in a WWTP. Such profiling can be based on simulated laboratory investigations mimicking

parameters within a specific WWTP or type of WWTP for each type of ENM. This is important for WWTPs that currently receive or expect to receive effluents from highly industrialised and urbanised areas, and secondly, would assist in identifying treatment processes or steps that could to be compromised due to antibacterial effects of ENMs. Consequently, such an approach would ensure that necessary informed quality assurance measures are taken in order to ensure the integrity of the treatment process. These may include, but are not limited to: bacterial population manipulation, prior chemical or physical manipulation of the effluent to increase the stability of ENMs, or stricter stabilisation of ENMs during the production phase of nanoproducts.

The antibacterial activity of ENMs in WWTPs can influence the quality of the water and sludge discharged into the environment, in terms of failure to eliminate some contaminants as well as the introduction of ENMs themselves to receiving environments. Based on current research results, it is difficult to gain any insights into possible microbial community changes due to continued exposure of ENMs to wastewater microorganisms or useful bacteria in agricultural soils and receiving water resources. Long-term effects of ENMs on bacterial populations, whether in soils or water, need to be carefully evaluated based on the assumption that the introduction of ENMs and partially treated chemicals (due to compromised bacterial viability) into the environment will occur over long periods and likely at sub-acute levels. It is therefore important that as we further deepen our understanding of the fate and behaviour of ENMs in WWTPs, we also take a "back to front" approach in order to investigate end-of-pipe implications for the receiving environment where ENMs are also a risk.

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