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5 **The minimum inhibitory concentration (MIC) assay with *Escherichia coli*: An**
6 **early tier in the environmental hazard assessment of nanomaterials?**
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Abstract

There are now over a thousand nano-containing products on the market and the antibacterial properties of some nanomaterials has created interest in their use as cleaning agents, biocides and disinfectants. Engineered nanomaterials (ENMs) are being released into the environment and this raises concerns about their effects on microbes in the receiving ecosystems. This study evaluated the bacterial toxicity of a wide range of nanomaterials with different surface coatings on *Escherichia coli* K-12 MG1655. The minimum inhibitory concentration (MIC) assay, which quantifies the threshold for growth inhibition in suspensions of bacteria, was used to rank the toxicity of silver (Ag), cupric oxide (CuO), cadmium telluride (CdTe) quantum dots, titanium dioxide (TiO₂), nanodiamonds and multi-walled carbon nanotubes (MWCNTs). Bacteria were exposed for 12 h at 37 °C to a dilution series of the test suspensions in 96-well plates. The precision and accuracy of the method was good with coefficients of variation < 10 %. In terms of the measured MIC values, the toxicity order of the ENMs was as follows: CdTe quantum dots ammonium-coated, 6 mg L⁻¹ > Ag nanoparticles, 12 mg L⁻¹ > CdTe quantum dots carboxylate-coated, 25 mg L⁻¹ > CdTe quantum dots polyethylene glycol-coated, 100 mg L⁻¹. The MIC values were above the highest test concentration used (100 mg L⁻¹) for CuO, TiO₂, nanodiamonds and MWCNTs, indicating low toxicity. The MIC assay can be a useful tool for the initial steps of ENMs hazard assessment.

Keywords: bioassay; ranking test; nanotechnology; tellurium; antimicrobial; metal dissolution

Introduction

Nanotechnology involves the manipulation of materials at the nanoscale in order to infer novel properties (Klaine et al., 2012). While engineered nanomaterials (ENMs) offer many potential benefits to society, there remains a need for safe (Gilbertson et al., 2015) and responsible innovation (Owen et al., 2009). Silver nanoparticles (NPs) are manufactured for their antimicrobial properties and are included in many consumable products (Morones et al., 2005). To date, the predicted environmental concentrations (PECs) of ENMs are low, with values typically in the $\mu\text{g L}^{-1}$ range in natural waters or much less (Lead et al., 2018). In terms of fate and behaviour, particle settling onto soils and sediments is also a concern (Meesters et al., 2016); resulting in the expose of natural microbial biofilms to ENMs.

Microorganisms provide key ecosystem services including aspects of nitrogen and carbon cycles in soils (Coleman et al., 2004). Microbial biofilms also form the base of food webs. Nonetheless, most of the available studies on the antibacterial effect of ENMs have used clinical isolates, including clinical strains of *Escherichia coli* and *Bacillus subtilis* (Yoon et al., 2007), as well as *Streptococcus aureus* (e.g., Xiu et al., 2014). For example, median minimum inhibitory concentrations (MICs) values of 7.1 mg L^{-1} and 200 mg L^{-1} have been reported for *E. coli* exposed to silver and cupric oxide NPs respectively (Bondarenko et al., 2013). Such laboratory studies show different degrees of bacterial sensitivity depending on the organisms and the type of ENM, but the mechanisms that cause it are not yet clear; although both contact toxicity and free ion toxicity have been suggested for Ag NPs (review by Reidy et al., 2013).

It was originally thought that the primary habitat of *E. coli* was the gut of host animals and humans; with faecal releases being an incidental route for the microbe to enter the environment. In fact, 'naturalised' strains of *E. coli* occur widely in soil and water (Winfield and Groisman, 2003). In the environment, *E. coli* retains its facultative adaptability and can process a range of soil nutrients including carbon, nitrogen, phosphorus and sulfur (Brennan et al., 2013). It is therefore an integral part of the natural microbiome. However, the effects of ENMs on microbial biodiversity in real ecosystems, or in complex biofilms, remains unclear. Additions of 1 mg L^{-1} of silver NPs to the overlying water showed no effects on microbial biodiversity of estuarine sediment (Bradford et al., 2009). In an investigation of silver and aluminium oxide NPs effects on soil microbes, a shift in soil microbial biodiversity was not observed, but changes to the microbial gene expression profile were detected (Fajardo et al., 2014).

74 While there remains some uncertainty around the environmental consequences of ENMs
75 on microbial ecosystem services, these organisms are nonetheless widely used in regulatory testing
76 for environmental hazard assessment (review by Crane et al., 2008). These include the
77 Organisation for Economic Co-operation and Development (OECD) 301, OECD 302A, 302B,
78 302C and OECD 308 biodegradation-related tests, as well as assays to measure microbial carbon
79 and nitrogen transformation in soils (OECD 216 and 217 respectively, see Crane et al., 2008).
80 Bacteria are also used incidentally for genotoxicity assessment due to their rapid cell division (e.g.,
81 in the Ames test), but there is no acute ecotoxicity test for bacteria exposed to chemicals, or ENMs
82 *per se* in the OECD testing strategy for the environment. A rapid tool would be especially useful
83 to provide an early demonstration of microbial ecotoxicity of ENMs and to rank ENMs of concern.

84 At present, regulatory ecotoxicity testing requires each new chemical substance to be tested
85 individually for hazard, including ENMs. There are some suggestions to rationalise testing,
86 including comparing ENMs with their nearest existing approved chemical substances (Crane et
87 al., 2008), and read-across approaches to deal with ENMs with surface coatings (Oomen et al.,
88 2014). Nonetheless, despite suggestions for amending the environmental testing strategy for ENMs
89 (Handy et al., 2012), the strategy remains expensive and laborious; but critically also without a
90 ranking tool that can be used to reduce the burden of regulatory work in an ecological context. In
91 addition, difficulties arise when one attempts to compare the results of toxicity tests conducted on
92 bacteria with ENMs, as no common testing protocols are being used in microbial ecotoxicology
93 research (different types of growth media, test suspension preparation methods, test vessels, test
94 duration and dosing, etc.). For product screening and regulatory ecotoxicology, a standardised
95 protocol that is robust and reproducible is therefore desirable.

96 There is a microbial toxicity test that has been standardised for the pharmaceutical industry.
97 The minimum inhibitory concentration (MIC) assay is the most commonly used technique in the
98 pharmaceutical testing of bactericidal activity (Wiegand et al., 2008). Specifically, the broth
99 dilution method consists of a bacterial inoculation in a liquid growth medium, in the presence of
100 different concentrations of the drug or antibiotic under test. This test has been recently adapted for
101 clinical samples of the oral pathogenic species *Streptococcus mutans* in the presence of silver,
102 silica and titanium dioxide ENMs (Besinis et al., 2014), but its utility as a simple routine
103 environmental test with *E. coli* and with a range of ENM chemistries has not yet been explored.

The primary aim of this study was to demonstrate the usefulness, from a nano-ecotoxicological perspective, of adopting the MIC assay for testing a range of ENMs with different chemistries under standardised exposure conditions to facilitate regulatory use. *Escherichia coli* K-12 MG1655 was chosen for the environmental reasons outlined above, but also because this strain is a model (Reinsch et al., 2012) for measurements of bacterial biomass and growth yields. A secondary aim was to evaluate any differences in the bacterial response to the presence of uncoated or coated (functionalised) ENM variants: carboxylate (-COOH), amine (-NH₂), ammonium (-NH₄⁺) and polyethylene glycol-coated types. Microscale (bulk) material counterparts or metal salts, as appropriate, were also tested.

Methods

Culture of microorganisms

The bacterial strain *Escherichia coli* K-12 MG1655 (DSM 18039) (Migula 1895) Castellani and Chalmers 1919 was purchased from the Leibniz Institute - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. This derivative of the K-12 strain has been cured of plasmids, virulence factors and phages (Guyer et al., 1981) and thus gives more consistent behaviour *in vitro*. It also has a high specific growth rate, μ (Hayashi et al., 2006).

In order to obtain reproducible inocula (Pirt, 1967), 10 mL nutrient broth was inoculated with a colony from a 24 h old nutrient agar slant. Subsequently, a 500 mL batch culture of *E. coli* K-12 MG1655 was grown for 12 h, then harvested by centrifugation at 4 °C, 10,000 x *g* for 30 min, washed and suspended in sterile saline (0.90 % (w/v) NaCl). This culture was diluted to a final optical density (*OD*) at 440 nm *c.* 0.90 (Jenway 7315 UV/Visible Spectrophotometer) and stored at - 80 °C until required for the experiments. The wavelength selected for *OD* calibration has a linear response of high sensitivity for short rods without any interference from the Soret peak of cytochrome *c* (Boden et al., 2010).

Experimental approach and the MIC assay

The current study investigated the toxicity of the following ENMs: silver metal (Ag); cupric oxide (CuO); cadmium telluride quantum dots (CdTe QDs); spherical and tubular forms of titanium

dioxide (TiO₂); nanodiamonds and multi-walled carbon nanotubes (MWCNTs), as compared to the equivalent metal salts or bulk controls. Mercuric chloride was used as the positive control for toxicity. The materials were uncoated and coated (functionalised) ENM variants as follow: carboxylate (COOH), amine (NH₂) / ammonium (NH₄⁺), or polyethylene glycol (PEG) functionalised ENMs for negative, positive and neutral surface functionalisation respectively. The precise details of how the coatings were synthesised and attached to the ENM core is commercially sensitive information of the suppliers, but for clarity we use the term ‘–NH₄⁺’ to mean an –NH₃ terminal ligand that has been ionised with H⁺ ions to achieve positive charge. The hypothesis was that uncoated ENMs may lead to observable toxicological effects, and that the surface-coatings of the ENMs may also influence the resulting toxicity. Ultrasound (sonication) was not used while preparing the test suspensions in order to prevent the detachment of the ENMs surface coatings from the uncoated materials and to avoid any possible ultrasonic-induced chemical changes to the test materials (Taurozzi et al., 2010). Critically, this approach also simplified the method for use as a standard regulatory test.

The minimum inhibitory concentration (MIC) assay allows the determination of the minimum dilution of a test suspension that gives complete inhibition of bacterial growth. The MIC assay experimental design is depicted in Supplemental Figure S1 with *n* = 6 replicate test plates. Initial experiments were also carried out in Erlenmeyer flasks to compare growth to that in 96-well plates. Growth was confirmed to be satisfactory in both test-vessels, although less absolute growth measured in 96-well plates (data not shown). Nonetheless, in the final protocol reported here, 96-well plates were chosen for their convenience over flasks.

In this work, analytical grade reagents or better were used. The E-basal salts (EBS) (Kelly and Syrett, 1964; Tuovinen and Kelly, 1973) as modified by Boden et al. (2008) was used as a minimal defined medium. This medium was also chosen as the least likely recipe to cause aggregation of ENMs in suspension. It was supplemented with 10 mM D-(+)-glucose (Sigma, ≥ 99.5 % purity) as the sole carbon and energy source. During the actual exposures in 96-well plates, test suspensions/solutions were diluted with the growth medium and the inoculum; with the resultant exposure medium referred to hereafter as ‘NaCl-EBS medium’. In this study, the term ‘test suspension’ or ‘stock suspension’ refers to the dispersion of ENMs or micron scale particles in a liquid, whereas the phrase ‘test solution’ refers to metal salts or other soluble substance in solution.

The final optimised MIC protocol for the experiments involved pipetting 200 μL of each test suspension/solution, at nominal concentrations ranging from 1.5 to 100 mg L^{-1} (prepared by serial dilution, see below), into 96-well microplates (flat-bottom sterile polystyrene, 0.415 mL capacity, Fisher brand) containing 20 μL of 10x strength EBS (sterilised just before plating, 0.2 μm Minisart Plus filter) and 22 μL of the inoculum above. Abiotic controls were run in parallel on each plate to account for the turbidity effect of the saline solution and for the turbidity caused by the test suspension or solution. A set of normal growth controls for growth in the absence of any test suspensions and positive controls (mercuric chloride, Sigma, 99 % purity) for complete growth inhibition (Boden and Murrell, 2011) were included. The 96-well plates were incubated (New Brunswick Scientific Model G25, Edison, USA) for 12 h at 37 $^{\circ}\text{C}$. The plates were allowed to shake at 130 rpm during the exposure period to ensure aerobic conditions in the medium. After the exposure period, the contents of the plates were pipette-mixed to re-suspend any material which may have deposited to the bottom of the wells. Immediately afterwards, OD_{440} was measured to determine growth using VersaMax microplate reader with SoftMax Pro 4.0 software (Molecular Devices, Sunnyvale, CA, USA), and appropriate correction for the turbidity controls. Glucose and carbon consumption were also determined (see below).

Test suspension preparation and characterisation

In order to clean and deionise glassware, all media bottles and glass stirring rods were washed overnight with 5 % (v/v) nitric acid (Fisher, Primer Plus Trace Metals Grade) trace analysis grade and rinsed three times with glass distilled water (distilled from ion-free ultrapure water, 18 M Ω resistance), then autoclaved for a 15 minute cycle at 121 $^{\circ}\text{C}$ and 15 psi. A sterile 1 g L^{-1} test suspension or solution, as appropriate of each material, was prepared by weighing 0.05 g (Sartorius BP 210) of powder and added to 50 mL of sterile glass distilled water. For metal salts, the amount of powder weighed was adjusted to account for the relative mass contribution of the metal species in the uncoated ENMs and bulk controls. All stock suspensions or solutions were stirred for 3 h (IKA-WERKE R015), set at speed 3. Then, a 1:10 dilution of the stock in sterile physiological saline (0.90 % NaCl) was prepared to achieve a working nominal concentration of 100 mg L^{-1} . The use of the saline prevented osmotic stress to the test organism during the exposures, while enabling a suitable dilution for the experiments. Subsequently, by a 50 % serial dilution in 0.90 % NaCl to the following nominal concentrations were made: 50, 25, 12, 6, 3, and 1.5 mg L^{-1} . Each

test suspension or solution was vortexed (Minishaker MS2 IKA) in between dilution steps to ensure homogeneity of the liquid for pipetting.

Details of the purity, primary particle size and surface area of the materials investigated are found in Table 1; together with the measured mean aggregate size and mean particle concentration in 0.90 % NaCl. Particle number concentration and particle size distribution in the 100 mg L⁻¹ nominal test suspensions were measured by nanoparticle tracking analysis (NTA) using a Nanosight LM 10 (Malvern Instruments, UK). Three sub-samples from each of the fresh stock suspensions (10 mL each) were vortexed for 10 s immediately before analysis. A capture timeframe of 10 s was used, with shutter and gain settings of 1390 and 200 respectively. Particle size distributions are reported as the particle number concentration, along with the hydrodynamic diameter in Table 1.

Dissolution of ENMs in the exposure media

The degree of metal ion dissolution in the NaCl-EBS medium was determined for silver, copper, cadmium, tellurium, and titanium containing materials by dialysis following the method of Besinis et al. (2014). These experiments were conducted at room temperature, pH 6.5, and with dialysis tubing with a molecular weight cut off 12 kDa (Sigma). The latter with a measured pore size by electron microscopy in our laboratory of < 2 nm. Dialysis bags were filled with 8 mL of the appropriate 100 mg L⁻¹ nominal test suspension or solution, and suspended in a 600 mL beaker containing 492 mL of the medium (in triplicate beakers). The experiments were consistently carried out under slow agitation, using a magnetic plate stirrer. Blanks with no test suspensions added to the dialysis bags were also included in each experiment. Samples of media (1 mL) were taken from the external compartment of the beaker at time zero, 30 min, 1, 2, 3, 4, 6, 12 and after 24 h. Samples were acidified by addition of 4 mL of 5 % (v/v) nitric acid. Samples of 0.5 mL media were also taken at each time point for pH measurements (Thermo Scientific Orion 2-Star Plus meter fitted with a Russell combination electrode). The contents of the dialysis bags were sampled both at the start (i.e., the stock used to fill it) and end of the experiment. The total metal concentrations of all the samples were analysed by inductively coupled plasma optical emission spectrophotometry (ICP-OES) or by inductively coupled plasma optical mass spectrophotometry (ICP-MS) using the Thermoscientific, X Series 2 (see below).

Total metal analysis

Total metal concentrations of silver, copper, cadmium, tellurium, titanium and mercury (Table 1) were measured in the NaCl-EBS medium using ICP-OES (Thermo Scientific, iCAP 7000 series). Prior to analysis, all samples were diluted 1:5 with 5 % (v/v) nitric acid. For titanium concentrations a 2:1 mixture of concentrated sulfuric acid to nitric acid (analytical grade reagents) was used (Mudunkotuwa et al., 2016). Acidified, matrix-matched ICP-OES standard metal solutions were used for calibrations and with sample blanks included every 10 samples in each run of the instrument. Internal standards of yttrium or indium were also included to correct for instrument drift during the analyses (Shaw et al., 2013).

Glucose determination

The concentration of unconsumed glucose in the plates was measured (GO-20 assay kit, Sigma) at the end of each experiment to ensure that growth had not been limited by the absence of glucose and/or loss of bioavailable glucose in the suspensions. The kit was used according to the manufacturer's instructions. The assay employs glucose oxidase to oxidise the glucose to gluconic acid and hydrogen peroxide. The latter reacts with a colour reagent to form a stable pink colour which is proportional to the original glucose concentration. Briefly, 140 μ L of the remaining volume from each experimental well, both test and control wells, were transferred to V-bottom microplates (V-bottom sterile polystyrene, 0.415 mL capacity, Fisher brand) which were centrifuged (2000 rpm for 10 min with a 2040 Rotors microplate centrifuge, Centurion Scientific Ltd, Ford, UK) to pellet the particles and the bacteria. Then, 4 μ L of the supernatant from each of the wells were removed to new flat bottom 96-well plates, and diluted with 36 μ L of water.

Biomass and growth yield determination

The correlation between *OD* at 440 nm and amount of biomass produced was determined from calibration curves prepared from suspensions of *E. coli* K-12 MG1655, diluted to various optical densities with spent culture and dried to constant weight at 85 °C. An *OD*₄₄₀ of 0.1 was experimentally found to correspond to 14 mg dry biomass L⁻¹ (data not shown). The growth yield (*Y_C*) as grams dry biomass per mole of carbon consumed was determined from the biomass produced and glucose consumed:

$$Y_c = \frac{\Delta \text{biomass}}{\Delta \text{glucose}}$$

Statistical analyses

Statistical analyses were carried out using IBM SPSS Statistics version 21. Following descriptive statistics, the Kolmogorov test was used to check for normality. Independent student *t*-tests and one-way analysis of variance (ANOVA, Tukey *post hoc* test) were used to check for significant differences amongst responses from within each test material and treatments (e.g., comparing absolute growth to each dilution effect) and then amongst treatments for the same material chemistry type (e.g., comparing percentage relative growth for carboxylate, ammonium, polyethylene glycol-coated nanodiamonds). All statistical analyses used a 95 % confidence limit, so that *p* values ≥ 0.05 were not considered statistically significant. The data are shown as mean $\pm$ standard error of the mean (S.E.M). The coefficient of variation expressed as a percentage was sometimes used to show the variability amongst plates for each test concentration and treatment. Dissolution curves were fitted using SigmaPlot version 12.5 by applying the hyperbola, single rectangular, 2 parameter equation on the raw data points.

Results

Behaviour of the nanomaterials in the test media

Incidental visual observations during the preparation of the stock dispersions identified that some materials tended to aggregate in the high ionic strength of the 0.90 % NaCl. There were some size effects with the Ag and CuO NPs appearing dispersed, whereas their micron scale counterparts (colloid silver, bulk CuO) tended to settle onto the bottom of the plates. There were also some apparent differences in the behaviour of the ENMs, depending on the coating. For example, the uncoated CuO and CuO-NH₄⁺ ENMs appeared well dispersed, whereas the CuO-COOH and CuO-PEG formed observable black aggregates in 0.90 % NaCl. In contrast, the coating effect was not the same for the CdTe QDs materials, with the CdTe-NH₄⁺ form changing to a greyish colour and sinking as visible aggregates. Visually, the nanodiamonds appeared as a good dispersion in 0.90 % NaCl, regardless of surface coating. Of the MWCNTs, the uncoated form proved to be more difficult to disperse in 0.90 % NaCl forming observable large aggregates.

The level of dispersion of the materials in 0.90 % NaCl was confirmed by NTA measurements of the hydrodynamic diameters and particle number concentrations (Table 1). In 0.90 % NaCl much larger hydrodynamic diameters than those reported in the manufacturers' information for the primary particle size were determined. For example, dry powders of the CdTe QDs had diameters in the 3 – 5 nm range, in contrast to much larger sizes (> 50 nm) as measured by NTA in 0.90 % NaCl (Figure 1a-c). The particle number concentrations in the dispersions of ENMs also generally decreased with the hydrodynamic diameters, consistent with some particle aggregation and settling in 0.90 % NaCl.

In terms of comparing ENMs with their micron scale counterparts, the larger hydrodynamic diameters were, as expected, mainly observed for the microscale materials (Table 1). For example, for Ag NPs the mean hydrodynamic diameter was less than 100 nm, but almost 200 nm in the micron scale colloidal silver equivalent (*t*-test, $p > 0.05$). However, the effect of ENM coatings on the mean hydrodynamic diameter was material-specific (Table 1). For example, the uncoated CuO ENMs (182 nm) showed no statistical significant difference in size relative to the coated forms of CuO NPs (ranges 120 – 140 nm for the coated CuO ENMs, ANOVA, $p > 0.05$). Particle size order as hydrodynamic diameters of CdTe coated QDs were also measured by NTA. Particle size by QD coating type increased in the following order: -NH₄⁺, -COOH and -PEG, with the ammonium and carboxylate forms (< 100 nm) statistically significantly smaller (ANOVA, $p < 0.05$) than the PEG form (> 150 nm); and bulk CdTe (> 100 nm) not different overall in particle size to that of the QDs (ANOVA, $p > 0.05$).

Confirming exposure in the NaCl-EBS medium

In addition to checking the state of dispersion and metal concentrations in the initial 0.90 % NaCl stock dispersions and solutions. Attempts were also made to measure the total metal concentrations in the NaCl-EBS medium at the start of the experiment compared to the expected nominal concentrations where relevant (Table 1). For the metal salts, the measured values were within 80 % of the nominal concentrations. For example, the measured concentrations as a percentage of the calculated total metal concentrations were: 88 % copper in CuSO₄, 79 % cadmium in CdCl₂, 98% tellurium in K₂TeO₃ and 91 % Hg in HgCl₂. The only exception to this was AgNO₃, which is known to form insoluble silver chloride particles in saline media, and thus the apparent dissolved silver was around 10 % of the expected nominal value. For all ENMs where measurement was

possible, the presence of total metal was confirmed. However, the measured metal concentrations in the NaCl-EBS media was less than the calculated total metal concentrations for the ENMs, with (% of nominal value): 5 % for Ag NPs; > 85 % for CuO NPs, 50 – 70 % for cadmium and tellurium in QDs; and 30 – 60 % for TiO₂-based ENMs. Similarly, for the bulk powders, the measured metal concentration were less than nominal, but still a readily detectable concentration that confirmed exposure had occurred (Table 1).

Dialysis experiments and release of total dissolved metals into NaCl-EBS medium

The total dissolved metal concentrations in the NaCl-EBS medium were measured over 24 hours during dialysis of the materials where it was technically possible. The maximum apparent dissolution rates were also calculated from the steepest initial phase of each dialysis curve (Table 1). Example dialysis curves are shown in Figure 2. The dialysis bags were filled with a total of 800 µg of each material, and yet the maximum dissolution rates were either at or below detection limits, or around 1-3 µg min⁻¹ for most substances (Table 1). Maximally, this equates to round 0.1 - 0.4 % of the metal in the material min⁻¹.

Silver regardless of form, showed the least release of total dissolved metal into the external compartment of the beakers compared to all the other metals tested, and with the lowest apparent initial dissolution rates (Table 1). After 12 h, both the bulk and the nano forms of silver showed some detectable metal concentrations in the external medium, but this remained low with no statistically significant differences between these materials (ANOVA, $p > 0.05$, Figure 2a). Of the substances made of silver, only the AgNO₃ showed a clear time-dependent appearance of total silver in the external media of the beaker (Figure 2a), being statistically higher than with the Ag ENMs or Ag bulk material by 12 hours (ANOVA, $p < 0.05$, Figure 2a).

Contrasting with the silver, the Cd and the Te metal salt controls as expected showed some solubility in the media, with the highest initial slopes on the dialysis curves of around 6 µg min⁻¹ (Table 1, equating to ~0.8 % min⁻¹). There were some material-type effects in relation to Cd and Te. The CdTe-QDs, regardless of coating, released less dissolved metal than the equivalent metal salts (Table 1, Figure 2b, c). There were also some small, but statistically significant coating effects, within the CdTe-QDs for the final total dissolved Cd concentrations in the beakers; with less Cd released from the CdTe-PEG material (ANOVA, $p < 0.05$, Figure 2b). Notably, this pattern in the coating effect for CdTe-QDs was not the same for the Te metal concentrations in the beakers,

with the Te concentrations being in the order: COOH > PEG > NH₄⁺ (Figure 2c); with the measured Te concentration being lower (ANOVA, $p < 0.05$, Figure 2c) in the beakers for the NH₄⁺ compared to the other coatings. The CdTe bulk material was even less soluble than the ENMs with no detectable increases of either Cd or Te metal concentrations in the beakers (Figures 2b and 2c).

Copper sulfate, like the Cd salt, caused some appearance of total dissolved metal in the external media of the beakers, although the rate of appearance of Cu was around 3 µg min⁻¹, approximately half that of CdCl₂ (Table 1). Similar to the findings with the CdTe materials, the CuO ENMs also showed some dissolution, but much less than the equivalent metal salt, and with the bulk CuO material showing the lowest Cu concentrations in the external media of the beaker (Table 1, Figure 2d). In terms of a coating-effect within the CuO ENMs (Figure 2e), there were some initial differences with faster appearance of Cu in the beakers from the CuO-COOH material, but this effect was lost within 6 hours, and thereafter no coating effect was observed for total Cu from the CuO ENMs (Figure 2e). Regardless of coating or not, the CuSO₄ gave the highest Cu concentrations in the beakers at the end of the experiment (ANOVA, $p > 0.05$, Figure 2d).

Minimum inhibitory concentration assay

Escherichia coli K-12 MG1655 in the absence of test materials showed normal growth, with a growth density of about 40 mg dry weight biomass per litre; whereas the positive control for toxicity (mercuric chloride), as expected, prevented growth at all the tested dilutions. Figure 3 shows the effect of the different test suspensions on bacterial growth using the MIC assay. The coefficient of variation (CV) indicated generally good reproducibility of this assay. The normal growth controls had a grand CV (average ± S.E.M, $n = 336$) of 6.1 % ± 1.0. The CV for the different materials were generally 10 % or much less, and summarised as follows by chemical substances: silver < 4 %; copper < 10 %; CdTe < 6 %; TiO₂ < 9 % and nanodiamonds < 5 %. The most variability was seen for the MWCNTs (24.9 % ± 2.7), especially at the highest test concentration.

The MIC values obtained from the growth curves are shown in Table 2. For several ENMs, even with a nominal exposure of 100 mg L⁻¹ no effects on growth were observed; or were modest at one or two of the higher exposure concentrations such that an MIC value could not be determined. This was the case for ENMs of cupric oxide, titanium dioxide, nanodiamonds and MWCNTs (MIC values above the maximum concentration used in the test, Table 2). However,

some of the CdTe and silver ENMs showed toxicity with a ranking as follows for the MIC when toxicity was observed in the experimental conditions used here: CdTe QD-NH₄⁺ > Ag NPs > CdTe QD-COOH > CdTe QD-PEG. Of the metal salts, AgNO₃ was consistently as inhibitory to *E. coli* K-12 MG1655 as mercuric chloride at all the concentrations tested (Figure 3a) and achieved the same MIC value as mercury (Table 2). Both CuSO₄ and CdCl₂ showed limited toxicity with MIC values greater than 100 mg L⁻¹. The K₂TeO₃ salt was slightly more toxic with an MIC value of around 50 mg L⁻¹ (Table 2).

In terms of material-type effects on bacterial growth, the Ag NPs were not as effective as AgNO₃, but nonetheless a nominal concentration of 12 mg L⁻¹ of Ag NPs or higher completely inhibited growth (Figure 3a). The micron scale Ag powder (colloidal silver) had limited biocidal properties and less than the nano form, with complete growth inhibition seen from 50 mg L⁻¹ or higher for colloidal silver. No particle coating experiments were performed with silver because coated versions of the Ag NPs were not available. For copper (Figure 3b), there was no growth difference for *E. coli* K-12 MG1655 between the bulk CuO and the uncoated CuO ENM from 1.5 to 50 mg L⁻¹ nominal concentrations. However, at the highest test concentration (100 mg L⁻¹) a material-type effect emerged with the bulk CuO stimulating bacterial growth, whereas the uncoated CuO ENM reduced the bacterial viability by approximately 20 % compared to the normal growth control (ANOVA, $p < 0.05$). For CuSO₄ exposure, statistically significant reductions in growth relative to the unexposed controls (ANOVA, $p < 0.05$) were only observed at nominal concentrations of 50 and 100 mg L⁻¹, with reductions of 20 and 50 % respectively (Figure 3b). The CuSO₄ was more toxic than the bulk CuO and of similar toxicity to the uncoated CuO ENM at the highest test concentration. However, there was no observable coating effect within the ENMs as none of the coatings showed growth inhibition.

The cadmium salt and the bulk CdTe equivalent to the quantum dots were not toxic to *E. coli* K-12 MG1655 (Figure 3c). For reasons related to the synthesis of the QDs there is no uncoated material. All the coated CdTe QDs studied were found to reduce bacterial viability, with growth inhibition being most severe in the presence of the ammonium coated QDs, followed by the carboxylate and PEG-coated forms. The tellurium metal salt displayed a linear concentration-related growth inhibition effect ($r^2 = 0.96$) up to the 25 mg L⁻¹ nominal concentration; and complete growth inhibition at 50 mg L⁻¹ and higher.

For the spherical TiO₂ ENMs (Figure 3d), only the TiO₂-NH₄⁺ and TiO₂-PEG forms inhibited growth compared to controls (ANOVA, $p < 0.05$), with the latter being more toxic. Neither the uncoated spherical TiO₂ ENM nor the bulk material were toxic. The same pattern of toxicity was observed with the tubular TiO₂ materials (compare Figure 3d and e). However, there was some evidence of a material-shape effect with the tubular TiO₂ being less toxic than the spherical form when toxicity was observed. Nanodiamonds generally had little or no effect on bacterial growth (Figure 3f), except for a statistically significant (ANOVA, $p < 0.05$), 20% reduction or less in growth compared to the controls for the -COOH and PEG-coated forms at the highest test concentration. In stark contrast to all other materials, the MWCNTs had a statistically significant (ANOVA, $p < 0.05$) and positive effect to enhance bacterial growth (Figure 3g), with possible exception of the MWCNT-COOH material that initially reduced growth at concentration up to 50 mg L⁻¹, but enhanced growth at 100 mg L⁻¹.

Yield measurements and glucose utilisation

In Figure 4, absolute biomass and growth yield are displayed for the CdTe QDs, spherical and tubular PEG-coated TiO₂ ENMs. For the QDs, the absence of biomass at the lethal doses was also reflected in zero carbon yields. At the sub-lethal doses of the QDs, a reduction in biomass with increasing test concentration was correlated to less measured carbon yield. For TiO₂ PEG-coated ENMs, the reduction in biomass at 100 mg L⁻¹ (for both NPs and NTs), and also at 50 mg L⁻¹ for NTs was reflected in a statistically significant reduction in measured yield relative to the growth control (ANOVA, $p < 0.05$).

Discussion

This work reports a modified MIC assay for determining the antibacterial effect of different commercial ENMs against the facultative microbe *E. coli* K-12 MG1655. For convenience and standardisation, the tests were carried out under fully oxidic conditions in 96-well plates; and without the need for complex sonication procedures for different particles. The MIC assay was rapid, with good reproducibility and showed differential sensitivity according to the materials tested. The test method could resolve growth inhibition due to material-type (e.g., nano *versus* bulk material or metal salt) and also coating-effects within some ENMs.

The utility of the MIC assay for testing ENMs

The MIC assay used here is reasonably quick with incubations of only 12 hours (e.g., overnight). It is simplified for ENMs by not having a bespoke target for dispersion of the stock suspensions, but instead standardised stirring to facilitate sub-sampling of freshly prepared stocks for dosing. From a practical perspective, the use of a 50 % serial dilution based on nominal concentrations is sufficient to obtain reproducible results and the MIC values obtained can be used to rank materials in order of toxicity. For example, here the MIC assay remained responsive to non-essential toxic metals, as illustrated by the expected high toxicity of mercury and silver (Table 2), while identifying Ag NPs as less hazardous (see below on metals). It also correctly identified the low toxicity of the TiO₂ and MWCNTs (Table 2), in keeping with our previous environmental work on other organisms such as fish (TiO₂, Boyle et al., 2013; MWCNTs, Boyle et al., 2014). The MIC was able to identify CdTe quantum dots as toxic, but also differentiate the hazard of coatings and the bulk material (see below). The assay therefore clearly has utility as for identifying materials of concern.

The test medium in the MIC assay used here is a defined salt solution that can be inoculated. Furthermore, since both the bacteria and the ENMs tend to settle in the high ionic strength medium used (as expected by DLVO theory, see Handy et al., 2008), the test organism remains exposed to the test substance. It is therefore not useful (or relevant) to collect media samples directly from the plates, as this would not reflect the true exposure at the bottom of the test wells. The MIC screening method is therefore not reliant on ‘water column’ concentrations of the test substance being maintained as in *Daphnia* or fish embryo tests (Shaw et al., 2016), or other routine ecotoxicity tests. In any event, the design used here also included turbidity controls on each plate so that the data is readily corrected for changes in optical density due to the particles.

Across the different materials studied, the reproducibility of the assay was not affected by the surface coating of the materials, or by testing the uncoated forms, the bulk or the equivalent metal salts. In terms of precision and accuracy, the MIC assay generally provided a consistent reproducible output across the different materials tested with coefficient of variation (CV) within all experiments of less than 10 %. MWCNTs presented the largest variability in the calculated percentage CVs (>15 %). The uncoated MWCNTs were especially poorly dispersed in the saline medium (visually observed). Given the hydrophobic nature of MWCNTs, some difficulty in solution handling is expected. However, adding positive or negative coatings to the MWCNT did

not resolve this problem. The use of appropriate dispersing agents for MWCNTs could improve the solution handling and reproducibility for these materials, but the use of dispersing agents are discouraged in the ecotoxicity testing strategy (see Handy et al., 2012).

The regulatory acceptance of any growth inhibition assay will require a demonstration of optimal growth conditions to ensure the protocol has maximum sensitivity and that the organisms are healthy at the start of the experiment. The microplates, volumes used, and the media were found to be sufficiently robust for the MIC assay. The density of biomass and yield for the growth of the controls (Figure 4) in the absence of any test suspensions were in accordance to, in-house, previously determined figures for this strain of *E. coli* K-12 MG1655. The bacterium was able to grow in the presence of various metal ions, and at elevated metal concentrations. The conditions in our experiments also ensured that glucose was not limiting to microbial growth, and thus the inhibitory effects of the substances reported above are associated with chemical/nanomaterial toxicity.

Overall, the MIC assay presented here has utility as a first tier or rapid bacterial ecotoxicity test to identify materials of concern. The utility comes from the simplicity, speed and reproducibility of the test; the inclusion of a Hg positive control; the ability to correctly identify ENMs as toxic or of low hazard (i.e., no false negatives); as well as differentiating coating-effects and metal salt-effects where toxicity occurs. This MIC assay could identify ENMs of concern, before proceeding to the relevant OECD aquatic toxicity or soil organism tests in the overall testing strategy. Qiu et al. (2017) even suggest that a modified MIC assay followed by gene expression could be a first tier in high-throughput screening assays for ENMs.

Toxicity of ENMs compared to dissolved metals

One concern for risk assessment is whether ENMs are more toxic than their equivalent metal salts, and thus might require additional regulation for environmental protection. In this study, mercuric chloride was used as a positive control because of its established toxicity to *E. coli* K-12 (Boden and Murrell, 2011), and the metal completely inhibited growth compared to unexposed controls as expected. Silver nitrate was also extremely toxic with an MIC value of 1.5 mg L⁻¹ (Table 2), similar to MIC values previously reported for Ag (7.1 mg L⁻¹, see Bondarenko et al., 2013). In contrast to the Hg and Ag salts, CdCl₂ were not toxic (Figure 3c) and CuSO₄ only inhibited growth at Cu concentrations of 50 mg L⁻¹ or higher (Figure 3b). These observations are in keeping with the

homeostatic control of both Cu and Cd via the P-type ATPases in *E. coli* (Rensing et al., 2000). The K_2TeO_3 showed intermediate toxicity (Table 2), but with a clear concentration-dependent inhibition of growth (Figure 3c). The tellurium metal salt was much more toxic than $CdCl_2$ (Figure 3c). Both these metals (and the metals from CdTe QDs, see Dumas et al., 2010) can be used in the respiratory transport chain of microbes. Any Cd ions generated during electron transfer could presumably be excreted by the ATPase. However, the reduction of tellurite by the respiratory chain causes significant oxidative stress and toxicity to microbes (Trutko et al., 2000). Interestingly, the ability of *E. coli* to maintain energy production as measured by the yield per mole of carbon (glucose) consumed was confirmed for the CdTe QDs (Figures 4a-c). Regardless of the molecular explanations, the overall, the ranking of the metal salts in the MIC assay (Table 2) were as expected, and similar to previous rankings of metal toxicity to *E. coli* (Harrison et al., 2004).

There are also concerns that ENM toxicity may be imparted by metal ion dissolution from the surface of the material, resulting in dissolved metal toxicity. In the current study, this appears not to be the case. For example, the Ag NPs were the most toxic of the ENMs tested (Table 2; Figure 3a), but showed only a background dissolution of silver (Figure 2a). In any event, the trace Ag ions released would be rapidly chelated as insoluble AgCl in the NaCl-rich media (see Besinis et al., 2014). The Cu containing ENMs showed some dissolution (Figures 2d and e), with the highest rate of Cu release from the CuO-COOH form ($3.67 \mu g \text{ min}^{-1}$, Table 1), but no toxicity was observed, except at one (high) concentration of the uncoated CuO ENMs (Figure 3b). In contrast, the bulk CuO stimulated growth (Figure 3b). This apparent hormesis is known for trace metals in *E. coli* and for Cu in several invertebrates; and possibly related to the early induction of metal resistance mechanisms in the cells (Stebbing, 1982). However, why this was observed with the bulk material and not the CuO ENMs is unclear.

The CdTe-QDs showed some toxicity with the ranking for the coating being $-NH_4^+ > -COOH > -PEG$ (Table 2). Although some dissolution of both Cd and Te was measured in the $\mu g \text{ l}^{-1}$ range from the dialysis experiments (Figures 2b and c; Table 1), the releases were not in the same order as the toxicity ranking of the CdTe QDs, suggesting dissolution was not the explanation. Regardless, for Cd dissolution, any metal release could not easily explain the toxicity of the QDs as $CdCl_2$ was not toxic in the experimental conditions here (Table 2). For the Te component of the CdTe QDs, the QDs were more potent in bacterial growth inhibition than the tellurium metal salt (Figure 3, Table 2), suggesting that dissolved tellurium species cannot explain

the observed QD toxicity. Unfortunately, there is only limited information on the mechanisms of toxicity of Te to microbes (review, Lemire et al., 2013). Nonetheless, the most toxic form of the QDs was the CdTe-NH₄⁺ (Figure 3c) with an MIC value of 6 mg L⁻¹ nominal concentration (0.03 mM Cd, 0.01 mM Te) (Table 2). One other study reports an MIC value for a different composition of CdTe QDs at 10 nM for *E. coli* K-12 MG1655 (Schneider et al., 2009).

Particle size and coating effects

The notion that particle size may influence toxicity has been widely debated and the environmental concern is that substances of the same chemical composition may be more toxic at the nano scale (Klaine et al., 2012). In the present study, the ENMs that showed toxicity (Ag NPs and the CdTe QDs) were all more toxic than their bulk material counterparts (Table 2). For example, the MIC values for Ag NPs and the (bulk) colloidal silver were 12 and 50 mg L⁻¹ respectively (i.e., the ENM was approximately 5 fold more toxic). The observed differences in the MIC values for Ag NPs cannot be explained by metal ion dissolution for the reasons above. In the case of the CdTe bulk material, no dissolution of either Cd or Te was observed (Figures 2b and c). However, direct contact toxicity where the particles settle onto the surface of the microbes (see Reidy et al., 2013) is possible. Interferences from other colloids in the media prevented the determination of the particle number concentrations directly in the NaCl-EBS medium, but particle settling was observed by eye during the experiments. In the case of the Ag NPs and colloidal silver, the particles and the bacteria sank to the bottom of the test plate wells together. Similar observations were made for the QDs, but with clearly faster settling of the NH₄⁺-coated QDs aggregates. It is likely that this settling enhanced the direct particle exposure of the microbes. NTA was possible in the 0.90 % NaCl solution (Table 1). In theory, any contact toxicity with the bacteria would be a function of the aggregate size and particle number concentration. Interestingly for the Ag bulk material in 0.90 % NaCl, there were fewer much larger aggregates than that of the Ag NPs, representing about a 5 fold increase of silver settling in the micron dispersion which was coincident with the increase in toxicity.

One central idea in the direct contact toxicity hypothesis of ENMs is that the particle coating imparts some chemical reactivity (i.e., toxicity) and/or bioavailability to the surface of the organism. Consequently, the type of surface coating may inform on toxicity, or influence the dispersion in such a way as to alter the exposure. In the present study, the surface coatings were

chosen to represent positive, neutral and negative charges. The surface ligands of the coatings studied (-COOH, -NH₄⁺, -NH₂, -PEG) are not inherently toxic to microbes, and are found widely in nature (Scott, 1968). Overall, there were no consistent trends of toxicity by surface-coating across the materials tested. The data does not support the general idea that toxicity is driven by the surface coating of the ENM. The only ENM that showed a clear coating-dependent effect on toxicity was the CdTe-QDs, with the -NH₄⁺ coating being the most toxic. However, the NTA in 0.90 % NaCl did not reveal any inherent trends in the aggregation behaviour of the different coatings that might explain this toxicity (Table 1). The results of the dialysis experiments (Figure 2) showed that the type of coating did influence the dissolution for both the coated CuO ENMs and the CdTe QDs; but this was not correlated with toxicity within the materials. For example, in both types of ENMs, the -COOH coating gave the greatest maximum dissolution rates (Table 1), but the -COOH coatings were not the most toxic (Table 2).

Finally, there are also concerns about particle shape, especially with high aspect ratio nanomaterials (Donaldson et al., 2010). In the present study, both TiO₂ particles and TiO₂ nanotubes were examined. The spherical TiO₂-PEG material showed greater toxicity than the nanotube form of the same material (compare Figures 3d and e). This difference in toxicity cannot be attributed to dissolution of Ti metal (not observed). However, even considering the limitations of NTA measurements with non-spherical particles, the spherical form made a greater number of larger aggregates than the equivalent TiO₂-PEG nanotube (Table 1), tentatively suggesting that settling behaviour contributed to this difference in toxicity arising from particle shape. Whatever the mechanism, both the spherical and nanotube forms decreased the yield per mole of carbon consumed (Figures 4d and e), suggesting interference with electron transfer in the respiratory chain. How this occurs and why the spheres had the strongest effect on yield of the two materials requires further investigation.

Conclusions and recommendations

This study has demonstrated the utility of a modified MIC assay that can detect the toxicity of a wide variety of ENMs with different surface-coatings, metal salts, and bulk materials; then rank them accordingly. The test method achieves many of the practical goals for routine use in regulatory ecotoxicology, being robust and with good reproducibility. The method is rationalised so that detailed and bespoke sample preparation is not required for each ENM, and particle settling

in the 96-well plate technique used here does not confound the exposure (low risk of false negative). Our recommendation is that the modified MIC assay can be adopted as a first tier in an ecotoxicity testing strategy for ENMs, and of the materials studied, the Ag NPs and CdTe QDs are identified as priority substances of concern.

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Declaration of interest

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Table 1. Characterisation of the stock suspensions and solutions in 0.90 % NaCl and in the NaCl-EBS medium, at the highest nominal test concentration of 100 mg L⁻¹.

Material (Supplier)	****Manufacturer's information	*Mean aggregate size in 0.90 % NaCl (nm)	*Mean particle concentration in 0.90 % NaCl (x10 ⁸ particles mL ⁻¹)	Calculated total metal concentration in NaCl-EBS medium		**Measured metal concentration in NaCl-EBS medium (mg L ⁻¹)	***Metal dissolution rate (µg min ⁻¹)
				(mg L ⁻¹)	(mM)		
Ag Bulk, CAS 7440-22-4 (Sigma-Aldrich 327085, Lot MKBR8201V)	Diameter, 2 - 3.5 µm; purity ≥ 99.9 % trace metal basis	198.33 ± 57.67	0.33 ± 0.03	84.3 Ag	0.77 Ag	0.42 Ag	< 0.01 Ag
Ag NPs, with PVP dispersant, CAS 7440-22-4 (Sigma-Aldrich 576832, Lot 7721KH)	Diameter, < 100 nm; purity, 99.5 % trace metals basis; Fisher sub-sieve sizer surface area, 5.0 m ² g ⁻¹	87.33 ± 2.4	3.72 ± 0.16	84.5 Ag	0.77 Ag	3.71 Ag	0.02 Ag
AgNO ₃ , CAS 7761-88-8 (BDH Chemicals)	0.10 M volumetric solution, certified	--	--	88.8 Ag	0.83 Ag	9.00 Ag	0.04 Ag
CuO Bulk, CAS 1317-38-0 (British Drug Houses Ltd)	Analar grade	258.67 ± 12.72	4.31 ± 0.96	66.4 Cu	1.05 Cu	55.54 ± 3.81 Cu	1.54 Cu
#CuO NPs uncoated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF1309121)	Diameter, 10 - 20 nm; surface area, 42 ± 2 m ² g ⁻¹	182.33 ± 13.38	3.06 ± 0.79	66.4 Cu	1.05 Cu	59.71 ± 1.82 Cu	3.42 Cu
#CuO NPs COOH-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	Diameter, 10 - 20 nm; surface area, 7.4 ± 0.5 m ² g ⁻¹	135.33 ± 31.55	1.60 ± 0.43	35.69 Cu	0.56 Cu	31.18 ± 0.47 Cu	3.67 Cu
#CuO NPs NH ₄ ⁺ -coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot 140114)	Diameter, 10 - 20 nm; surface area, 6.1 ± 0.5 m ² g ⁻¹	120.33 ± 32.33	1.82 ± 0.08	43.16 Cu	0.67 Cu	41.51 ± 1.11 Cu	1.81 Cu

#CuO NPs PEG-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	Diameter, 10 - 20 nm	126.33 ± 9.26	1.13 ± 0.23	24.07 Cu	0.38 Cu	22.26 ± 0.02 Cu	2.00 Cu
CuSO ₄ ·5H ₂ O, CAS 7758-99-8 (Sigma-Aldrich 31293, Lot SZBC0170V)	Purity, 99 - 102 %	--	--	65.5 Cu	1.03 Cu	57.90 ± 0.26 Cu	3.71 Cu
CdTe Bulk, CAS 1306-25-8 (Sigma-Aldrich 256544, Lot MKBK6448V)	Diameter, < 250 µm; purity, ≥ 99.99 % trace metal basis	116.67 ± 17.48	0.77 ± 0.03	39.3 Cd 44.3 Te	0.35 Cd 0.35 Te	12.77 ± 0.82 Cd 14.28 ± 1.03 Te	< 0.01 Cd < 0.01 Te
#CdTe QDs COOH-coated, CAS 1306-25-8 (PlasmaChem GmbH, Lot YF140402)	Diameter, 3 -5 nm	76.67 ± 2.19	10.66 ± 0.21	35.4 Cd 5.81 Te	0.31 Cd 0.016 Te	24.00 ± 0.60 Cd 3.99 ± 0.16 Te	4.31 Cd 4.41 Te
#CdTe QDs NH ₄ ⁺ -coated, CAS 1306-25-8 (PlasmaChem GmbH, Lot YF140402)	Diameter, 3 -5 nm	65.33 ± 14.65	6.19 ± 1.61	52.1 Cd 28.3 Te	0.46 Cd 0.22 Te	33.92 ± 0.18 Cd 13.62 ± 0.26 Te	3.74 Cd 2.30 Te
#CdTe QDs PEG-coated, CAS 1306-25-8 (PlasmaChem GmbH, Lot YF140402)	Diameter, 3 -5 nm	157.33 ± 12.67	2.71 ± 0.07	49.5 Cd 9.23 Te	0.44 Cd 0.07 Te	28.99 ± 0.18 Cd 5.32 ± 0.07 Te	3.00 Cd 3.81 Te
CdCl ₂ , CAS 10108-64-2 (Sigma-Aldrich 202908, Lot MKBM1769)	Purity, 99.99 % trace metal basis	--	--	38.7 Cd	0.34 Cd	30.6 ± 0.14 Cd	6.97 Cd
K ₂ TeO ₃ , CAS 7790-58-1 (Hopkin and Williams Ltd)	97 % minimum K ₂ TeO ₃	--	--	46.4 Te	0.36 Te	46.79 ± 1.38 Te	6.71 Te
TiO ₂ Bulk, CAS 13463-67-7 (Acros Organics 277370010, Lot A0224336)	Purity, 98.0 - 100.5 %	301.00 ± 27.61	28.95 ± 2.75	51.83 Ti	1.08 Ti	35.57 ± 4.65 Ti	< 0.01 Ti
#TiO ₂ NPs uncoated (PlasmaChem GmbH, Lot YF1310291)	Diameter, 10 -20 nm; surface area, 98 ± 10 m ² g ⁻¹	36.00 ± 1.15	4.66 ± 0.50	51.83 Ti	1.08 Ti	31.14 ± 1.36 Ti	< 0.01 Ti

#TiO ₂ NPs COOH-coated, (PlasmaChem GmbH, Lot YF140402)	Diameter, 10 - 20 nm	186.67 ± 21.11	0.72 ± 0.17	^	^	^^	^^
#TiO ₂ NPs NH ₄ ⁺ -coated (PlasmaChem GmbH, Lot YF140114)	Diameter, 10 - 20 nm	58.33 ± 12.45	3.24 ± 0.09	^	^	^^	^^
#TiO ₂ NPs PEG-coated (PlasmaChem GmbH, Lot YF140402)	Diameter, 10 - 20 nm	145.00 ± 2.52	8.83 ± 0.97	^	^	^^	^^
#TiO ₂ NTs uncoated (PlasmaChem GmbH, Lot YF140402)	Aspect ratio, 1:5; surface area, 123 ± 10 m ² g ⁻¹	24.67 ± 4.09	2.18 ± 0.34	51.83 Ti	1.08 Ti	15.55 ± 1.07 Ti	< 0.01 Ti
#TiO ₂ NTs COOH-coated (PlasmaChem GmbH, YF140402)	Aspect ratio, 1:5; surface area, 20 ± 2 m ² g ⁻¹	54.33 ± 21.3	2.71 ± 0.38	^	^	^^	^^
#TiO ₂ NTs NH ₄ ⁺ -coated (PlasmaChem GmbH, Lot YF140402)	Aspect ratio, 1:5; surface area, 41 ± 4 m ² g ⁻¹	53.00 ± 7.1	3.55 ± 0.27	^	^	^^	^^
#TiO ₂ NTs PEG-coated (PlasmaChem GmbH, Lot YF140402)	Aspect ratio, 1:5; surface area, 11 ± 1 m ² g ⁻¹	45.00 ± 11.72	3.16 ± 0.32	^	^	^^	^^
#Nanodiamonds COOH-coated, CAS 7782-40-3 (PlasmaChem GmbH, Lot YF1310311)	Diameter, 4 - 6 nm; surface area, 180 ± 15 m ² g ⁻¹	59.00 ± 5.57	4.71 ± 0.12	--	--	--	--
#Nanodiamonds NH ₄ ⁺ -coated, CAS 7782-40-3 (PlasmaChem GmbH, Lot YF140114)	Diameter, 4 - 6 nm; surface area, 289 ± 10 m ² g ⁻¹	55.33 ± 17.03	2.98 ± 0.70	--	--	--	--
#Nanodiamonds PEG-coated, CAS 7782-40-3 (PlasmaChem GmbH, Lot YF140114)	Diameter, 4 - 6 nm; surface area, 244 ± 20 m ² g ⁻¹	68.00 ± 5.13	3.12 ± 0.23	--	--	--	--

#MWCNTs uncoated, CAS 308068-56-6 (Nanocyl 7000, Batch A2105)	Aspect ratio, 1:100; purity, 98 % carbon by weight; surface area, $213 \pm 5 \text{ m}^2 \text{ g}^{-1}$	28.67 ± 2.33	0.31 ± 0.04	--	--	--	--
#MWCNTs COOH-coated, CAS 308068-56-6 (Nanocyl 3151, Batch 131205)	Aspect ratio, 1:100; purity, 98 % carbon by weight; surface area, $284 \pm 2 \text{ m}^2 \text{ g}^{-1}$	42.33 ± 9.02	0.63 ± 0.17	--	--	--	--
#MWCNTs NH ₂ -coated, CAS 308068-56-6 (Nanocyl 3152, Batch 131205)	Aspect ratio, 1:100; purity, 98 % carbon by weight; surface area, $253 \pm 10 \text{ m}^2 \text{ g}^{-1}$	42.33 ± 7.22	0.42 ± 0.08	--	--	--	--
#MWCNTs PEG-coated, CAS 308068-56-6 (University of Manchester)	Aspect ratio, 1:100; purity, 98 % carbon by weight; surface area, $26 \pm 3 \text{ m}^2 \text{ g}^{-1}$	64.67 ± 14.84	4.00 ± 0.50	--	--	--	--
HgCl ₂ , CAS 7487-94-7 (Sigma-Aldrich M1136, Lot 071K1201)	Purity, 99 %	--	--	83.0 Hg	0.41 Hg	$75.29 \pm 0.21 \text{ Hg}$	^^

#Material supplied via the NANOSOLUTIONS Project; Quantum dots (QDs); Polyethylene glycol (PEG); Multi-walled carbon nanotubes (MWCNTs); Nanoparticles (NPs); Nanotubes (NTs); Polyvinylpyrrolidinone (PVP); *Data are the mean aggregate hydrodynamic diameters and mean particle concentration from particle size distribution measurements made by NTA using the Nanosight LM10. Values are mean $\pm$ standard error of the mean (S.E.M) ($n = 3$ measurements of the dispersion); **Data are means $\pm$ S.E.M ($n = 3$ replicates) of total measured metal concentration by ICP-OES; ***Dissolution rate calculated from dialysis experiments in triplicate; ****Unless otherwise stated, Brunauer–Emmett–Teller (BET) surface area values (mean $\pm$ one standard deviation, $n = 3$) provided *via* the NANOSOLUTIONS Project; --Data irrelevant to the chemical; ^Not possible to calculate from the manufacturers' information on material composition; ^^Not measured.

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Table 2. Minimum inhibitory concentration (MIC) values in mg L⁻¹, following *Escherichia coli* K-12 MG1655 exposure to a dilution series of the test materials with nominal concentrations of these materials from 1.5 to 100 mg L⁻¹ in 96-well plates. ‘> 100’ shows no detection of a MIC value for that material at all the tested concentrations. ‘> 100 *’ shows no detection of a MIC value for that material at all the tested concentrations, with more than 20 % bacterial growth inhibition relative to the negative growth control at the 100 mg L⁻¹ nominal test concentration, and statistical significant difference in absolute growth (biomass production in mg dry biomass L⁻¹) from the normal growth control (ANOVA, $p < 0.05$) at the 100 mg L⁻¹ nominal test concentration.

Chemical	ENMs uncoated	ENMs COOH- coated	ENMs NH ₄ ⁺ - coated [§]	ENMs PEG- coated	Bulk form	Metal salt ^{§§}
Ag	12	--	--	--	50	1.5
CuO	> 100 *	> 100	> 100	> 100	> 100	> 100 *
CdTe	--	25	6	100	> 100	> 100 Cd 50 Te
Spherical TiO ₂	> 100	> 100	> 100 *	> 100 *	> 100	--
Tubular TiO ₂	> 100	> 100	> 100 *	> 100 *	> 100	--
Nanodiamonds	--	> 100 *	> 100	> 100	--	--
MWCNTs	> 100	> 100	> 100	> 100	--	--
Hg	--	--	--	--	--	1.5

Engineered nanomaterials (ENMs); carboxylate functional coating (COOH); ammonium functional coating (NH₄⁺); polyethylene glycol functional coating (PEG); multi-walled carbon nanotubes (MWCNTs); [§] amine functional coating (NH₂) for MWCNTs only; ^{§§} metal salts used were AgNO₃, CuSO₄·5H₂O, CdCl₂, K₂TeO₃, HgCl₂; -- not applicable.

Figure Legends

Figure 1. Particle size distribution (bin sizes are hydrodynamic diameter in nm) measured by Nanoparticle Tracking Analysis (Nanosight LM10) of the cadmium telluride quantum dots; (a) carboxylate-coated, (b) ammonium-coated, (c) polyethylene glycol-coated, at the 100 mg L⁻¹ nominal concentration in 0.90 % (w/v) NaCl. Data presented as mean $\pm$ S.E.M, from 3 sub-samples of each prepared stock. Note, the particle number concentrations are $\times 10^6$ particles mL⁻¹

Figure 2. Dialysis curves showing the release of total dissolved metal ions from test suspensions/solutions over a 24 h period when suspended in NaCl-EBS growth media at pH 6.5 for: (a) silver, (b) cadmium, (c) tellurium, (d) uncoated copper oxide, and (e) coated copper oxide materials with their appropriate controls. The measured metal concentrations have been normalised to a starting dialysis concentration of 100 mg L⁻¹ of test material. Data are means $\pm$ S.E.M ($n = 3$) separate beakers for each material type and control beakers. Curves were fitted using a hyperbola, single rectangular, two parameter equation (SigmaPlot 13). Equations for the curve fits are reported in the supplement (Table S1).

Figure 3. The effect of the different test material nominal concentrations on bacterial growth, expressed as a percentage relative to the normal growth control (absence of test suspensions), using the MIC assay for (a) silver, (b) copper, (c) cadmium telluride quantum dots, (d) spherical titanium dioxide, (e) tubular titanium dioxide, (f) nanodiamonds, (g) MWCNTs. Data are mean $\pm$ S.E.M ($n = 6$). Different letters indicate significant differences amongst the relative tested concentrations (ANOVA, $p < 0.05$). Complete absence of letters means no statistical difference between the types of material within the test. ‘*’ refers to statistical significant difference in calculated biomass from the growth control at that particular concentration.

Figure 4. Biomass (mg dry weight biomass L⁻¹) and growth yield (g per mole of carbon consumed) from test materials exposure and normal growth control (no test suspension) for (a) CdTe QDs COOH-coated, (b) CdTe QDs NH₄⁺-coated, (c) CdTe QDs PEG-coated, (d) TiO₂ NPs PEG-coated, (e) TiO₂ NTs PEG-coated. Statistical significant difference from the growth control is represented with ‘*’ for both biomass and yield with ANOVA, $p < 0.05$. Complete absence of figure bars signifies no measurable biomass and/or yield.

Figure 1.

(a) CdTe QDs COOH

(b) CdTe QDs NH₄⁺

(c) CdTe QDs PEG

800 **Figure 2.**

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808 Figure 3.

Figure 3. (cont.)

824 **Figure 4.**

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