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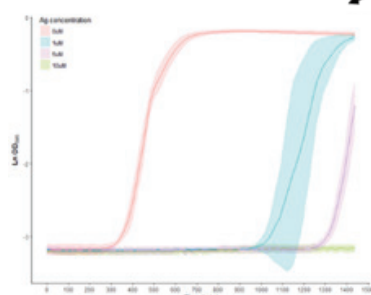
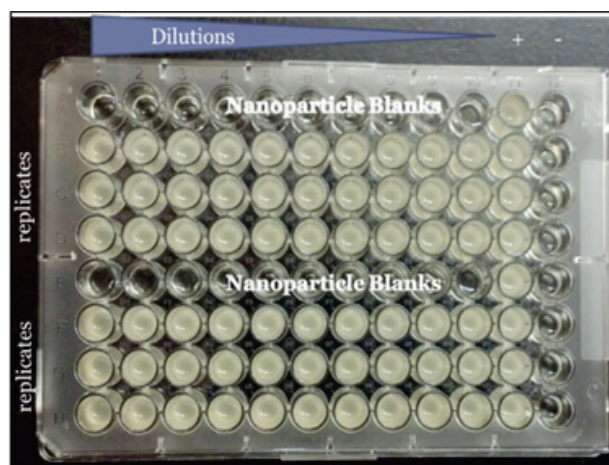
Environmental Consequences of Nanotechnologies

Determining the Bacterial Cytotoxicity of Silver Nanomaterials

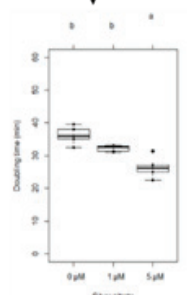
Scientific Operating Procedure Series: Toxicity (T)

Paul B. Schweiger, Brittany Twibell, Marshal Blank,
and Alan J. Kennedy

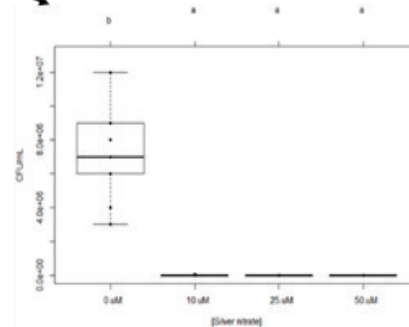
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**MIC Assessment
and Growth Kinetics**



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Determining the Bacterial Cytotoxicity of Silver Nanomaterials

Scientific Operating Procedure Series: Toxicity (T)

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Final Report

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Abstract

Materials at the nanoscale have physiochemical properties that differ from their bulk material. Consequently, the growing use of nanomaterials in industrial and consumer products raise important questions about their environmental fate. The potential cytotoxic effects on microbial communities is an important safety and material life cycle consideration, as microorganisms play a vital role in environmental nutrient cycling, influence health, and disease of plants and animals, including humans. This Scientific Operating Procedure (SOP) describes a set of methods to determine the cytotoxic effects of nanomaterials on bacteria. Initial growth effects, including minimal inhibitory concentration (MIC), can be monitored by a standardized microplate broth dilution assay when the nanomaterials stay suspended and do not inhibit optical density (OD) measurements. When these requirements are not met, a solid agar dilution plate MIC assay can be used whereby toxicity is determined by colony counts on agar plates. A standard minimum bactericidal concentration (MBC) assay is also detailed and can elucidated the mode of action of nanomaterials that have an MIC.

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Preface

This work was performed under Task 3: Environmental Health PE 0603728A, the contract number was W912HZ-15-2-0032. The Project Title was “Advancing Carbon Nanomaterials-Based Device Manufacturing through Life Cycle Analysis, Risk Assessment and Mitigation.” This program was funded by the Engineer Research and Development Center (ERDC), U.S. Army Corps of Engineers (USACE), Environmental Laboratory (EL). This work was directed by Rishi J. Patel, Senior Research Scientist at Missouri State University’s Jordan Valley Innovation Center. The technical monitor was Jerry Miller (CEERD-EL-EMJ).

The work was performed by the Environmental Risk Assessment Branch (EPR) of the Environmental Processes and Engineering Division (EP), ERDC-EL. At the time of publication, Dr. William M. Nelson was Branch Chief, CEERD-EPR, Mr. Warren P. Lorentz was Division Chief, CEERD-EP, and Dr. Elizabeth A. Ferguson was the Technical Director, Military Materials in the Environment. The Deputy Director of ERDC-EL was Dr. Jack E. Davis and the Director was Dr. Ilker R. Adiguzel.

COL Ivan P. Beckman was Commander of ERDC, and Dr. David W. Pittman was the Director.

Acronyms and Abbreviations

AgNPs	Silver Nanoparticles
ANOVA	Analysis Of Variance
CFUs	Colony Forming Units
CFU/ml	Colony Forming Units/Milliliter
CI	Confidence Interval
DoD	Department of Defense
EL	Environmental Laboratory
EHS	Environmental Health and Safety
EPE	Environmental Processes and Engineering
EPR	Environmental Risk Assessment Branch
EQT	Environmental Quality and Technology
ERDC	Engineer Research Development Center
g	Gram
HQUSACE	Headquarters, U.S. Army Corps of Engineers
mL	Milliliter
MBC	Minimum Bactericidal Concentrations
MIC	Minimum Inhibitory Concentration
Mg/L	Milligrams/Liter
nm	Nano Meters
NP	Nanoparticals
OD	Optical Density
PPE	Personal Protective Equipment
QA/QC	Quality Assurance/Quality Control
SOP	Scientific Operating Procedure
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency

1 Introduction

This Scientific Operating Procedure (SOP) describes the assessment of the toxicity of silver nanoparticles (AgNPs) on bacteria and was developed jointly under The Safe and Rapid Development of Nanotechnologies Partnership and the Advanced and Additive Materials: Environmental Sustainability research program.

This SOP describes how to determine the toxicity of AgNPs using bacteria as an indicator. Bacteria are exposed to a gradient concentration of nanoparticles (NPs) and growth is monitored over time (typically 16–24 hours). Toxicity is rapidly observed, and is indicated by growth defects including growth inhibition. Determination of a minimum inhibitory concentration (MIC) and/or minimum bactericidal concentration (MBC) allows the toxicity of the NP to be compared to known toxins.

1.1 Background

Silver has long been known to be toxic to bacteria (Alexander 2009). Today, various AgNPs are used not only in medical settings, but in consumer products (mobile phones, refrigerators, clothes, plasters, toothbrushes, cosmetics, etc.) to inhibit microbial growth (Chernousova and Epple 2013). These nanomaterials can then be released into the environment, raising concerns about the toxicity of these products (Geranio et al. 2009; Gottschalk and Nowack 2011).

Materials within the nanoscale range have high surface area and chemical and physical properties that differ from the same type of bulk material, this lends to the question of potential impacts for humans and ecosystems from nanomaterials (Reinhart et al. 2010; USEPA 2010). These different physiochemical properties of nanomaterials can potentially alter their cytotoxicity. For example, AgNPs release ions by oxidation, unless they are fully insoluble. These ions are often implicated in cytotoxic effects. The release of Ag ions is dependent on NP size, surface functionalization, temperature, and solvent composition (Ho et al. 2010; Kittler et al. 2010; Liu and Hurt 2010; Liu et al. 2010; Lok et al. 2007; Zhang et al. 2011; Zook et al. 2011). Alternatively, cytotoxicity has been linked to particle-specific effects that result from the particle composition (Griffitt et al. 2008; Griffitt et al. 2009; Kennedy et al. 2014; Laban et al. 2010; Sotiriou et al.

2011). This SOP is not intended to be used to determine the mechanism of toxicity. It is, however, important to identify potential cytotoxicity of different AgNPs. These data can be used to inform decisions based on potential impacts to humans and ecosystems. The effect of nanomaterials on the growth and viability on the bacterial community is a key part of an environmental life cycle assessment of chemicals, as bacteria are important factors in nutrient cycling and community structure.

The growth behavior of the model organism *Escherichia coli* K12 is well established, this makes it a common first choice microbe for cytotoxicology studies. Various silver species are known to be toxic to *E. coli*. However, minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) vary dramatically (Choi et al. 2008; Greulich et al. 2012; Jain et al. 2009; Kim et al. 2011; Martínez-Catañón et al. 2008; Suresh et al. 2011). For example, reported MIC values range from as low as 0.5 mg/L (Greulich et al. 2012) to no toxicity up to 150 mg/L (Martínez-Catañón et al. 2008). However, these results are not strictly comparable due to different experimental conditions, different silver species, different media compositions, and different bacterial strains used. This highlights the importance of standardized testing for AgNPs testing for initial bacterial cytotoxicity screens. This SOP should be performed to determine if AgNPs induce changes in growth behavior or complete inhibition of growth (i.e., MIC) in *E. coli* K12. When cell growth is completely inhibited, the mode of action of the AgNP can be determined with a MBC assay. This assay first exposes cells to the AgNP at the MIC concentration, and to several concentrations above the MIC. After exposure, the AgNP is then removed and the cells allowed to grow. If the cells are dormant they will start growing, indicating a bacteriostatic mode of action. If they do not grow, then the AgNP is considered bactericidal. These data can be used as an initial indicator that the NP is potentially deleterious to environmental microorganisms that could lead to disruptions in community structure or function.

1.2 Objectives

The primary objective of this task was to develop robust SOPs for investigating the environmental health and safety (EHS) related properties of nanomaterials.

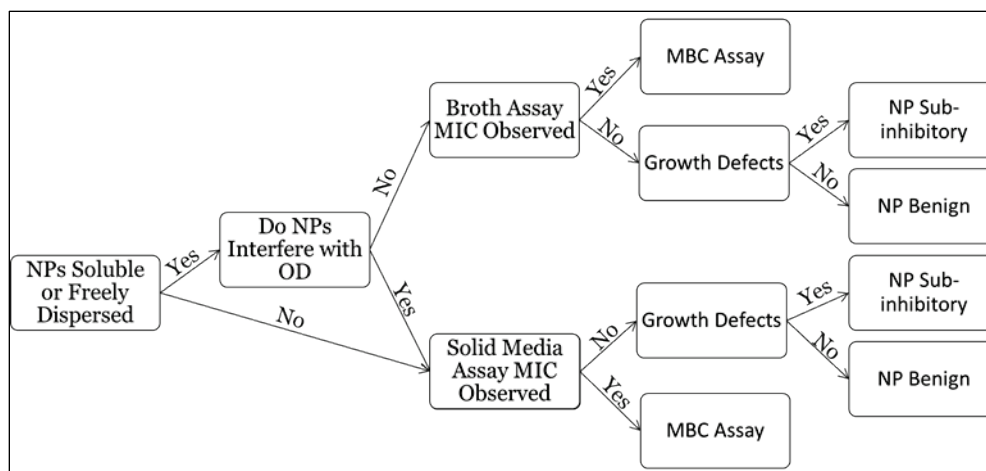
1.3 Approach

There was a need to develop methods specific to determining if nanomaterials used in novel sensing technologies have physiochemical properties that differ from bulk material that would uniquely impact microbial communities and thus nutrient cycling, which in turn could have cascading ecosystem impacts. Therefore, procedures and supportive data were developed to determine if AgNPs are cytotoxic to bacteria by monitoring growth during NP exposure using 96-well plates to allow rapid information about the toxicity of the NP under study.

1.4 Scope

This SOP is used to rapidly investigate the potential cytotoxicity of AgNPs to bacteria using *E. coli* K12 as a model, but could be modified to examine cytotoxicity of other prokaryotes (Figure 1). It is applicable to NPs that are freely dispersed in broth or agar interacting with the bacterial population.

Figure 1. Procedural decision tree summarizing this document.



2 Materials and Apparatus

2.1 Materials

- Deionized water: for rinsing flasks and preparing the medium
- Agar for solid medium
- Erlenmeyer flasks: for preparing the medium
- Petri plates (polystyrene, 100 mm x 25 mm)
- AgNPs to be tested
- Personal protective equipment (PPE): gloves, safety glasses, lab coat
- 1.5–2.0 ml polypropylene centrifuge tubes
- 1.5–2.0 ml polypropylene tube holders
- 96 well plates
- Tryptone
- yeast extract
- NaCl
- $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$
- KH_2PO_4
- NH_4Cl
- MgSO_4
- Glucose
- CaCl_2
- Thiamine
- HCl for pH adjustments of the medium
- NaOH for pH adjustments of the medium
- *Escherichia coli* K12
- 0.22–0.45 μm syringe filters
- 20 ml syringes
- 10 μl , 200 μl , 1000 μl micropipettes
- 10 μl and 300 μl multichannel pipettors

2.2 Apparatus

- Temperature-controlled water bath: to stabilize the temperature of agar-containing media
- Temperature-controlled water bath sonicator: to ensure even distribution of NPs in the medium
- Centrifuge for 1.5–2.0 ml microfuge tubes
- Dry air incubator for bacterial culture
- Shaking incubator for bacterial culture

- Microplate reader capable of shaking and temperature control
- Autoclave
- pH meter
- Computer with Microsoft Excel (or equivalent alternative),
R programming language and RStudio

3 Procedure

The following protocol describes media preparation for solid and liquid medium containing AgNPs. An undefined, nutrient-rich medium or a defined minimal salts medium may be used. Each petri plate contains 25 ml of solid agar medium supplemented with AgNPs. All components of the medium are sterilized except for the AgNPs, these cannot be autoclaved or filter-sterilized without causing damage to the AgNPs, changing their physiochemical properties, or changing their concentration. Sterility is checked by incubating the AgNP in growth medium for 24 hours. The absence of growth is considered sterile. If growth occurs, the nanomaterial may need to be sterilized by filtration, autoclave, dry heat, or chemical treatment. Following sterilization, the physiochemical properties and concentration of the AgNP should be determined, this is beyond the scope of this SOP.

3.1 Specimen preparation

3.1.1 Preparing the medium

- Outgrow *E. coli* K12 from frozen stocks.
- Setup bacterial cultures in 96 well plates or on solid medium, inoculating a uniform cell number into each well or petri plate.
- Co-culture *E. coli* K12 with the nanomaterial.
- Measure cell growth by optical density (OD) or by counting colony forming units (CFUs).
- Report any impact of the test material on cell growth and determine MIC when observed.
- If an MIC is observed, determine an MBC.

3.1.2 Lysogeny broth

- Rinse a clean 250 ml Erlenmeyer flask with deionized water.
- Add 75 ml of deionized to the Erlenmeyer flask.
- Add 1.0 g tryptone, 0.5 g yeast extract, 1.0 g NaCl to the Erlenmeyer flask and fully dissolve.
- Adjust the pH to 7.0 by adding HCl or NaOH.
- Bring the volume up to 100 ml with deionized water.
- Autoclave the flask at 121 °C for 20 minutes.

3.1.3 M9 minimal broth medium

- Rinse a clean 250 ml beaker with deionized water.
- Add 75 ml of deionized to the beaker.
- To make a 5x M9 solution add 6.4 g $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 1.5 g KH_2PO_4 , 0.25 g NaCl, 0.5 g NH_4Cl and fully dissolve.
- Adjust the pH to 7.0 by adding HCl or NaOH.
- Bring the volume up to 100 ml with deionized water.
- Autoclave the flask at 121 °C for 20 minutes.
- Autoclave 80 ml of deionized water.
- While autoclaving, filter sterilize (0.22–0.45 μm filter) 10 ml of the following solutions: 1M MgSO_4 , 20% glucose, 1M CaCl_2 , 1M thiamine.
- To make a 1x M9 + B1 solution combine the following sterile components and mix thoroughly 80 ml deionized water, 20 ml 5x M9, 200 μl 1M MgSO_4 , 2 ml 20% glucose, 10 μl 1M CaCl_2 , 100 μl thiamine.

3.1.4 Solid medium

- Preheat water in the sonicator and the warm water bath to 55 °C.
- Rinse a clean 250 ml Erlenmeyer flask with deionized water.
- Add 1.5 g of agar into the flask.
- To make lysogeny broth, see 3.1.2. To make M9 see 3.1.3.
- Wearing PPE, place the sterile solid medium in a 55 °C water bath to keep the agar liquid.
- Add the desired amount of NPs to the medium. If the AgNP is a powder, disperse in sterile deionized water or buffer by sonicating for two minutes 55 °C while swirling prior to addition to the medium.
- Mix the medium containing the AgNP by sonication for two minutes at 55 °C while swirling to ensure even distribution of the NPs within the medium.
- Immediately pour 25 ml into sterile petri plates and solidify at ambient temperature.
- Once the plates are poured, immediately rinse the flask with warm water to prevent residual agar from solidifying to the sides and discard. (Note if AgNPs are present, collect waste in a designated container and dispose of following applicable local, state, and federal regulations).

3.1.5 Bacterial outgrowth

- Streak out *E. coli* onto solid medium without AgNPs.
- Culture overnight at 37 °C in a dry heat incubator.

- Pick one well isolated culture and inoculate 5 ml of medium.
- Culture overnight at 37 °C in a shaking incubator.

3.2 Cytotoxicity assays

3.2.1 Microdilution, MIC assays

3.2.1.1 Standardized inoculum preparation

To standardize the inoculum used for MIC assays, preliminary standard plate counts are used to determine the colony forming units per milliliter (CFU/ml) of *E. coli* K12 in the growth medium (either lysogeny broth or M9+B1) after a 16–24-hour incubation period at 37 °C.

- *E. coli* K12 was outgrown in triplicate as described in section 3.1.5.
- The OD at 595 nm was determined.
- Dilute the cultures to an OD of 1.0 with outgrowth medium to normalize the cell numbers.
- Serially dilute the normalized cultures from 10^1 to 10^7 in 1.5 ml microfuge tubes using the outgrowth medium.
- Divide petri plates (two per culture; six total) containing solid medium (see 3.1.4) of the same type as the outgrowth medium into four quadrants.
- 5 µl from each dilution is spotted three times in one quadrant of the solid medium.
- Incubate the plates 16–24 hours at 37 °C in a dry heat incubator.
- Count colonies of the dilutions that produce between 5–15 colonies and calculate the CFU/ml at an OD of 1.0 using Equation 1.

$$\frac{CFU}{mL} = \frac{CFU \cdot \text{dilution factor}}{0.005 mL} \quad (1)$$

- The final cell number added to each assay is adjusted by diluting cells outgrown overnight to an OD₅₉₅ of 1.0 so that the CFU/ml is known and can be controlled. A standard final cell concentration is adjusted to 5.0×10^5 CFU/ml in each assay.

3.2.1.2 AgNP optical interference determination

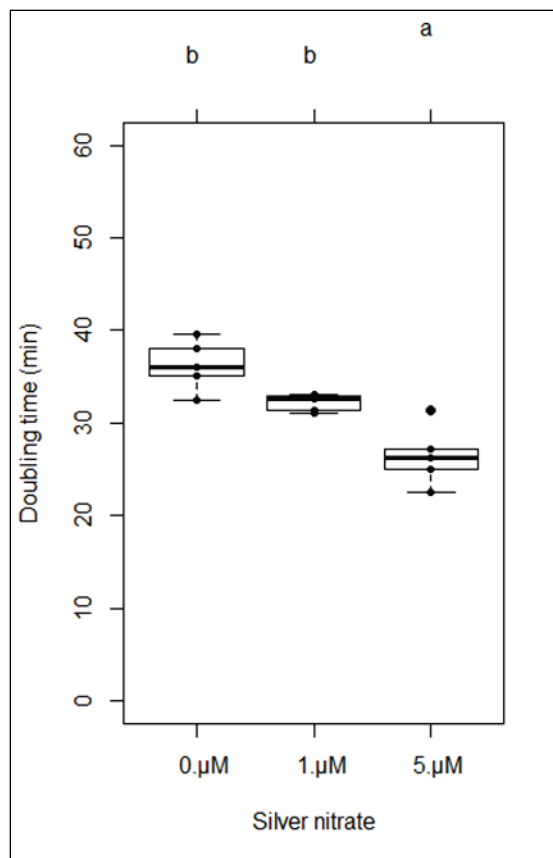
- The extent of the optical interference of the AgNP should be determined empirically by mixing the AgNP with the growth medium at the desired test concentrations.

- Compare the OD of the AgNP, at all concentrations, to that of blanks that do not contain AgNPs, using the wavelength to be used to monitor cell growth (e.g., 595 nm, 600nm, 630 nm). Particles and concentrations that have an OD >0.1, or if OD is unstable, should be considered to have optical interference and not be used in broth MIC assays. In these cases, an MIC can be determined by solid media MIC assays (see 3.2.1.4).

3.2.1.3 *Broth microdilution MIC determination*

- Prepare a dilution of the culture to achieve a cell density of 10^6 CFU/ml as for the MIC assay (see 3.2.1.1).
- Prepare concentrations of the nanomaterial using the growth medium as a diluent. If the AgNP is a powder, disperse in sterile deionized water, or buffer by sonicating for two minutes while swirling prior to addition to the medium. Use the highest possible concentration that still allows OD to be monitored. This concentration will vary depending on the exact NP used. From the highest possible concentration, prepare 500 μ l each of ten serial ten-fold dilutions.
- In the top row (row A) of a microtiter plate, mix 100 μ l of the nanomaterial concentrations with 100 μ l of growth medium. This will serve as a blank to correct for background absorbance by the medium and NPs (Figure 2).
- To wells B1, C1, and D1, add 100 μ l of the 10^6 CFU/ml cell suspension to the highest dilution of NPs.
- Repeat previous step for the other nine AgNP dilutions adding the mixture to the adjacent wells (e.g., B2, C2, D2).
- As a positive control, add 100 μ l of the 10^6 CFU/ml cell suspension and 100 μ l of medium wells A12, B12, C12, and D12.
- As a negative control, add 200 μ l of the medium to A11, B11, C11, and D11. (Note that this is only uses half of a 96-well microplate. More dilutions can be used or a second nanomaterial may be tested on the same plate).
- Place the microplate into a microplate reader capable of maintaining a temperature of 37 °C with shaking.
- Monitor growth for 16–24 hours, taking OD₅₉₅ every five minutes
- After incubation, subtract the blank values for each row from the average absorbance and plot the OD₅₉₅ vs. time to create growth curves.

Figure 2. Example of doubling time distribution plot. Concentrations that share letters in common are not statistically different. Here, 1 μM silver nitrate does not influence growth, whereas 5 μM AgNO_3 is inhibitory but not lethal.



- Compare the growth to the positive and negative control, noting delays in log-phase growth, changes in doubling time (T_d), and final OD_{595} .
Note: If there is growth in the negative control or the blank wells the assay must be repeated.
- Calculate exponential phase doubling time (T_d) using Equation 2.

$$T_d = \frac{\log 2 \cdot t}{\log N - \log N_o} \quad (2)$$

Where, N is the ending OD of log-phase growth, N_o is the starting OD at log-phase growth, and t is the time between N and N_o

Alternatively, T_d can be calculated using Equation 3, calculating the growth rate constant (k) in exponential phase and then determining T_d by using Equation 4

$$k = \frac{\ln N}{\ln N_0} \quad (3)$$

$$Td = \frac{\ln 2}{k} \quad (4)$$

- It is recommended that Td and final OD_{595} be determined automatically using an R programming language script (see Analysis section 6.3) or a similar automated method to increase throughput and consistency.
- The lowest concentration of nanomaterial, where no growth is observed, is considered the MIC. If an MIC is observed, an MBC should be determined (see 3.2.2). If an MIC is not observed and no growth delays or defects are observed, the nanomaterial is likely to be benign to the growth of *E. coli* K12 and no further testing presented in this SOP is necessary. However, if an MIC is not found, but growth defects are observed, this indicates a sub-lethal effect of the nanomaterial.

3.2.1.4 MIC determination on solid medium

- Prepare solid medium containing the nanomaterial (see 3.1.4). Use the highest possible concentration. This concentration will vary depending on the exact NP used. Prepare a minimum of four solid media concentrations containing ten-fold serial dilutions of the NP to give concentrations of NPs.
- Prepare a dilution of the culture to achieve a cell density of 5.0×10^5 CFU/ml as described for the MIC assay (see 3.2.1.1).
- Spot 5 μ l of the 5.0×10^5 CFU/ml on solid medium without NPs in triplicate, as described in section 3.2.1.1.
- To the same plate spot 5 μ l of dilution medium in triplicate
- Continue spotting 5 μ l on the NP dilution plates in a similar fashion.
- Incubate the plates 16–24 hours at 37 °C in a dry heat incubator
- Count colonies of the dilutions that produce between 5–15 colonies and calculate the CFU/ml using Equation 5.

$$\frac{CFU}{mL} = \frac{CFU \cdot \text{dilution factor}}{0.005 \text{ mL}} \quad (5)$$

Note: The goal is to observe small colonies that are not confluent for accurate cell counts. Optimization of conditions with an untreated control may be necessary.

- Compare the growth to the unexposed, positive control, and negative controls that do not contain cells.

Note: If there is growth in the negative controls, or no growth in the positive controls, the assay must be repeated.

The lowest concentration of nanomaterial where no growth is observed is considered the MIC. If an MIC is observed, an MBC should be determined (see 3.2.2). If an MIC is not observed and no growth defects are observed, the nanomaterial is likely to be benign to the growth of *E. coli* K12 and no further testing presented in this SOP is necessary. However, if an MIC is not found but growth defects such as lower CFU/ml than the positive control are observed, this indicates a sub-lethal effect of the nanomaterial.

3.2.2 MBC assay

- Prepare a dilution of the culture to achieve a cell density of 10^6 CFU/ml as for the MIC assay (see 3.2.1.1).
- Prepare concentrations of the nanomaterial at 1X, 5X, 10X, and 20X the observed MIC value using the growth medium as a diluent. If not possible to make all concentrations due to limitations in stock solution concentration, make dilutions of the nanomaterial as high as possible above the MIC.
- In the top row of a microtiter plate, mix 100 μ l of the nanomaterial concentrations with 100 μ l of the 10^6 CFU/ml cell suspension in duplicate.
- As a positive control, add 100 μ l of the 10^6 CFU/ml cell suspension and 100 μ l of medium to two of the remaining wells in the top row of the microtiter plate.
- As a negative control, add 200 μ l of the medium to the last two wells in the top row of the microtiter plate.
- Incubate the microplate in a microplate reader in the same way as for the MIC assay (see 3.2.1.3).
- After 16–24 hours (this time should be similar to the MIC assay endpoint time), serially dilute the cells in the top row of the microtiter plate to 10^{-7} in columns B–H. To do this, mix 180 μ l medium with 20 μ l from the well directly above and mix thoroughly by trituration.
- Spot 5 μ l of each dilution (10^0 – 10^{-7}) on petri plates containing agar in triplicate using a micropipette as described in section 3.2.1.1.
 - Alternatively, a slot pin replicator can be used.
- Incubate the plates 16–24 hours at 37 °C in a dry heat incubator.

- Count colonies of the dilutions that produce between 5–15 colonies and calculate the *CFU/ml* using Equation 6.

$$\frac{CFU}{mL} = \frac{CFU \cdot \text{dilution factor}}{0.005 \text{ mL}} \quad (6)$$

Note: The goal is to observe small colonies that are not confluent for accurate cell counts. Optimization of conditions with an untreated control may be necessary. In cases where the bacterium spreads during growth (e.g. *Proteus*), it will be necessary to use standard plate counts.

- The lowest concentration of nanomaterial where no growth is observed is considered the MBC. If a MBC is not observed, then the nanomaterial is considered bacteriostatic rather than bactericidal.

3.3 Analysis

Analysis begins after 16–24 hour incubation of the cultures described in 3.2. A minimum of one 96-well plate can be used for 3.2.1.3 and 3.2.2 (Table 1). Two petri plates are also needed for 3.2.1.4 and 3.2.2. The analysis workflow presented requires the ability to create .csv files and run an R script with minimal modification to what is presented below. Refer to the individual package manual for more information about particular packages used and other functionalities not presented here.

Table 1. Example of a 96-well broth microdilution MIC assay plate setup.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 9	Dilution 10	Positive control	Negative control	Blank
B	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 9	Dilution 10			Experimental
C	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 9	Dilution 10			Experimental
D	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 9	Dilution 10			Experimental
E	Dilution 11	Dilution 12	Dilution 13	Dilution 14	Dilution 15	Dilution 16	Dilution 17	Dilution 18	Dilution 19	Dilution 20	Positive control	Negative control	Blank
F	Dilution 11	Dilution 12	Dilution 13	Dilution 14	Dilution 15	Dilution 16	Dilution 17	Dilution 18	Dilution 19	Dilution 20			Experimental
G	Dilution 11	Dilution 12	Dilution 13	Dilution 14	Dilution 15	Dilution 16	Dilution 17	Dilution 18	Dilution 19	Dilution 20			Experimental
H	Dilution 11	Dilution 12	Dilution 13	Dilution 14	Dilution 15	Dilution 16	Dilution 17	Dilution 18	Dilution 19	Dilution 20			Experimental

3.3.1 Analysis of Broth Microdilution MIC

- Import the time course OD data of from all wells, including all controls and blank wells in 3.2.1.3.
- Subtract the corresponding background OD recorded in the blank wells from the wells with the corresponding OD data.
- Arrange these data with the time and well numbers horizontally across the top row with the data below in columns and save as a .csv file (Table 2).

Table 2. Example corrected OD data.

time	B3	C3	D3	E3
0	0.042	0.043	0.045	0.042
10	0.042	0.042	0.045	0.042
20	0.042	0.042	0.045	0.042
30	0.041	0.042	0.045	0.042
40	0.042	0.042	0.045	0.042
50	0.042	0.042	0.045	0.042
60	0.042	0.042	0.045	0.042
70	0.042	0.042	0.045	0.042
80	0.042	0.042	0.045	0.042
90	0.042	0.042	0.045	0.042
100	0.042	0.042	0.045	0.042
110	0.042	0.042	0.045	0.042
120	0.042	0.042	0.045	0.042

- Install R and RStudio (<https://www.r-project.org/>, <https://www.rstudio.com/products/RStudio/>) (R Development Core Team 2008; RStudio Team 2015)
- Open RStudio and install the dependent packages: dplyr, ggplot2, growthcurver (Sprouffske and Wagner 2016), multcomp (Hothorn et al. 2008), reshape2:

```
install.packages("dplyr")
install.packages("ggplot2")
install.packages("growthcurver")
install.packages("multcomp")
install.packages("reshape2")
```

- The following annotated script can be run to calculate doubling time, plot doubling time distributions, and plot growth curves, and perform statistical analysis:

```
#Load libraries
library("dplyr")
library("ggplot2")
library("growthcurver")
library("multcomp")
library("reshape2")

#Generate doubling times
#Import the optical density data with the background absorbance
#subtracted out
ODreads<-read.csv("File name of OD vs time data.csv")
```

```
#Calculate the growth rate constant (k), doubling time (Td)
plate <- SummarizeGrowthByPlate(ODreads)

#show the calculated growth metrics in RStudio
print(plate)

#Save file with k, Td (i.e. t_gen) growth data
write.csv(plate, file = "Growthcurver output file name.csv")

#example of output file (Table 3)
```

Table 3. Example Growthcurver data output.

	sample	k	n0	r	t_mid	t_gen	auc_l	auc_e	sigma	note
1	B3	0.742932	4.24E-05	0.018243	535.5865	37.9961	671.9152	669.69	0.01815	
2	C3	0.76697	6.73E-05	0.017513	533.3909	39.57924	695.3384	692.785	0.015539	
3	D3	0.789461	2.89E-05	0.019204	531.9169	36.09465	716.895	714.965	0.011105	
4	E3	0.777473	2.26E-05	0.019735	529.3681	35.12308	707.9906	706.16	0.010007	

```
#Plot doubling times with statistical groupings:
#Arrange the Td data from the output file just created with the
#nanoparticle concentration on the top row and the doubling times are
#given below as follows and save as a csv file (Table 4):
```

Table 4. Example doubling time.

0 μ M	1 μ M	5 μ M
37.9961	33.02275	27.25897
39.57924	31.43474	31.44191
36.09465	32.87233	22.54758
35.12308	32.58178	26.30967
32.50458	31.03513	25.06169

```
Import the Td data in the file just created:
Td=read.csv("Td file name.csv",header=TRUE)
```

```
#Rearrange the data into two columns
Tdmelt<-melt(Td)
```

```
#statistical analysis
anova<-aov(value~variable, data=Tdmelt)
tuk<-glht(anova, linfct = mcp(variable = "Tukey"))
summary(tuk)
cld(tuk)
```

```
#Box-and-whisker plot of Td with data points overlaid in a strip chart
(Figure 3)
```

```
plot(cld(tuk), xlab="Silver nitrate", ylab="Doubling time (min)",
ylim=c(0,60))
stripchart(value~variable, vertical=TRUE, data=Tdmelt,
method='overplot', add = TRUE, pch = 20, col = 'black')
```

The following annotated scripts will generate growth curves with 95% confidence intervals:

First, natural log transform the OD data previously saved as a .csv file. Again, save these data as a .csv file (Table 5).

Table 5. Example of Ln transformed OD data.

Time	Temperat	B3	C3	D3	E3	F3
0	37	-3.17009	-3.14656	-3.10109	-3.17009	-3.27017
10	37	-3.17009	-3.17009	-3.10109	-3.17009	-3.27017
20	37	-3.17009	-3.17009	-3.10109	-3.17009	-3.27017
30	37	-3.19418	-3.17009	-3.10109	-3.17009	-3.27017
40	37	-3.17009	-3.17009	-3.10109	-3.17009	-3.27017
50	37	-3.17009	-3.17009	-3.10109	-3.17009	-3.27017
60	37	-3.17009	-3.17009	-3.10109	-3.17009	-3.27017

```
#Import the Ln transformed data in the file just created
LnOD <- read.csv("LnOD file.csv")
```

```
#create and load a plate map (Table 6:
platemap <- read.csv("platemap.csv")
```

Table 6. Example plate map.

Well	Ag concentration
B3	0uM
B4	1uM
B5	5uM
B6	10uM
C3	0uM
C4	1uM
C5	5uM
C6	10uM
D3	0uM
D4	1uM
D5	5uM
D6	10uM
E3	0uM
E4	1uM
E5	5uM
E6	10uM
F3	0uM
F4	1uM
F5	5uM
F6	10uM

```
# Reshape the data. Instead of rows containing the Time, Temperature,
# and data for each well, rows will contain the Time, Temperature, a
# well ID, and the data for that Well.
shape <- melt(LnOD, id=c("Time", "Temperature"),
variable.name="Well", value.name="OD595")

# Add information about the experiment from the plate map. For each
# well defined in both the reshaped data and the platemap, each
# resulting row will contain the absorbance measurement as well as the
# additional columns and values from the platemap.
annotated <- inner_join(shape, platemap, by="Well")

# Save the annotated data as a .csv file for storing, sharing, etc.
# (Table 7.
write.csv(annotated, "your file name.csv")
```

Table 7. Example of annotated data save file.

	Time	Temperat	Well	OD595	Ag.concentration
1	0	37	B3	-3.17009	0uM
2	10	37	B3	-3.17009	0uM
3	20	37	B3	-3.17009	0uM
4	30	37	B3	-3.19418	0uM
5	40	37	B3	-3.17009	0uM
6	50	37	B3	-3.17009	0uM
7	60	37	B3	-3.17009	0uM
8	70	37	B3	-3.17009	0uM
9	80	37	B3	-3.17009	0uM
10	90	37	B3	-3.17009	0uM

```
# Calculate the 95% confidence interval for your growth curves
conf_int95 <- function(data) {
  n <- length(data)
  error <- qt(0.975, df=n-1) * sd(data)/sqrt(n)
  return(error)}

```

- The following script will group the data by the different experimental variables and calculate the sample size, average OD, and 95% confidence limits around the mean among the replicates. It will also remove all records where the Ag concentration is not provided and is listed as NA. Lastly, growth curves with 95% confidence intervals will be generated (Figure 4).

```
stats <- annotated %>% group_by(Ag.concentration, Time) %>%
summarise(N=length(OD595), Average=mean(OD595),
CI95=conf_int95(OD595)) %>% filter(!is.na(Ag.concentration))
```

```

plot<- ggplot(data=stats, aes(x=Time, y=Average,
color=Ag.concentration)) +
  geom_ribbon(aes(ymin=Average-CI95, ymax=Average+CI95,
fill=Ag.concentration),
  color=NA, alpha=0.2) +
  geom_line(show.legend = FALSE) + theme_classic() +
  scale_x_continuous(breaks =
c(0,100,200,300,400,500,600,700,800,900,1000,1100,1200,1300,14
00,1500)) +
  labs(x=expression(bold("Time (min)")), y=expression(bold('Ln OD'
[595])))

plot + scale_fill_discrete(breaks=c("0uM", "1uM", "5uM", "10uM")) +
  theme(legend.position=c(0.1,0.9))

```

#Example of a growth curve plot (Figure 3). The average is the solid line
 #with the shaded 95% confidence interval shown as a shaded ribbon. It #is
 quickly seen that both 1 μ M and 5 μ M Ag delay growth, whereas 10 μ M is
 inhibitory and is the MIC. Taken together with the doubling #time data,
 this suggests that 10 μ M Ag is inhibitory, and 1 μ M and 5 μ M Ag cause
 growth defects such as protracted lag times (1 μ M and 5 μ M) and decreased
 growth rates (5 μ M).

Figure 4 shows that both 1 μ M and 5 μ M Ag delay growth, whereas 10 μ M
 is inhibitory and is the MIC. Taken together with the doubling time data,
 this suggests that 10 μ M Ag is inhibitory, and 1 μ M and 5 μ M Ag cause
 growth defects such as protracted lag times (1 μ M and 5 μ M) and
 decreased growth rates (5 μ M).

Figure 3. Example of growth curves generated. *Solid line*, average OD; *ribbon*, 95% confidence interval (CI).

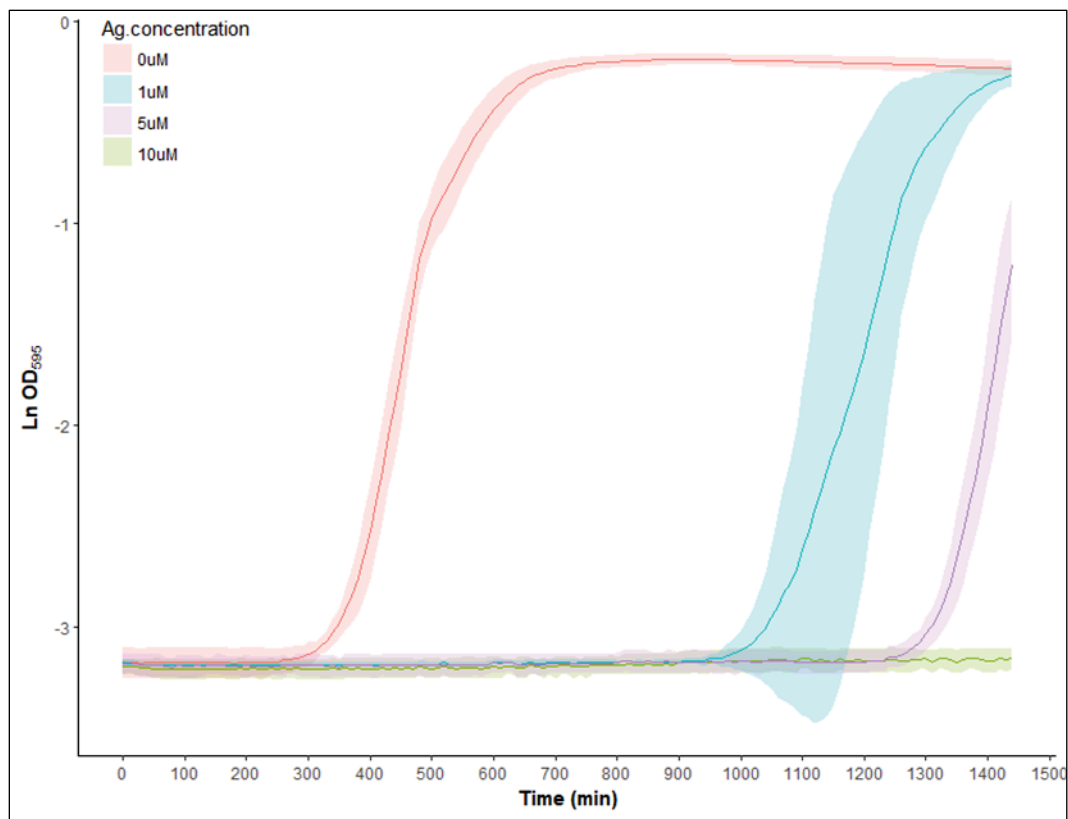
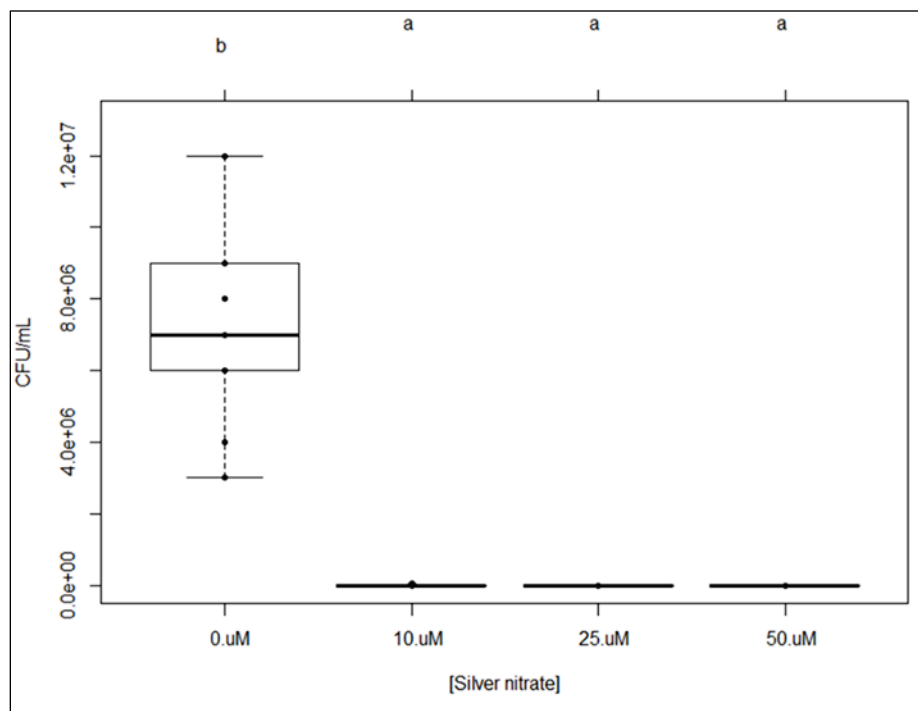


Figure 4. Example of generated CFU/ml Plot. Concentrations that share letters in common are not statistically different.



3.3.2 Analysis of MIC and MBC plate counts

- Calculate the CFU/ml for each spot as described as in section 3.2.1.4 and 3.2.2.
- Create a table with the Ag concentration in the first row and the CFU/ml listed underneath for each spot. Save as a .csv file (Table 8):

Table 8. Example of CFU count data.

0 uM	10 uM	25 uM	50 uM
7000000	10000	0	0
9000000	10000	0	0
12000000	0	0	0
30000000	0	0	0
80000000	0	0	0
90000000	0	0	0
70000000	0	0	0
40000000	0	0	0
60000000	0	0	0

- Install R and RStudio (<https://www.r-project.org/>, <https://www.rstudio.com/products/RStudio/>)
- Open RStudio and install the dependent packages: dplyr, ggplot2, growthcurver, multcomp, reshape2.

```
install.packages("multcomp")
install.packages("reshape2")
```
- The following annotated script can be run to create and analyze CFU/ml distribution plots:

```
#Load libraries
library("multcomp")
library("reshape2")
```

```
#Import the CFU/ml data in the file just created:
CFU=read.csv('CFU.csv',header=TRUE)
```

```
#Rearrange the data into two columns
CFUmelt<-melt(CFU)
```

```
#statistical analysis
anova<-aov(value~variable, data=CFUmelt)
tuk<-glht(anova, linfct = mcp(variable = "Tukey"))
summary(tuk)
cld(tuk)
```

Although statistically similar to 25 μM and 50 μM Ag, the 10 μM Ag concentration had cell growth on the spot plates. When performing a solid medium MIC assay (3.2.1.4), 25 μM and 50 μM concentrations inhibited cell growth, and are considered inhibitory with the MIC being 25 μM . If a statistically significant ($p \leq 0.05$) decrease in CFU/ml is observed without complete inhibition of growth an MIC would not be observed. However, the NP should be considered deleterious to growth. If an MIC is observed, a MBC assay (3.2.2) can be done. If Figure 5 represented a MBC assay, then the 25 μM Ag should be considered the MBC since an average of $\sim 10^4$ cells were observed for 10 μM Ag (Table 7) and the Ag nanomaterial would be considered bactericidal. If an MIC is observed but not an MBC, the NP is considered bacteriostatic.

4 Reporting

Each of the functional assays in section 3 requires a different method of analysis, key results, and quality assurance/quality control (QA/QC) considerations. Thus, broth and solid media-based assays are given as a subsection within the overall assay.

4.1 Analysis of results

Raw OD data are analyzed to determine doubling times and monitor growth defects (e.g., increased lag-phase, changes in doubling time, lower final OD, and abolishment of cell growth) using Microsoft Excel and the R programming language (3.3.1). First, OD is corrected for the background absorbance of the medium or medium containing AgNPs. Doubling times are calculated, and the distributions are plotted in a box-and-whisker plot with the raw data points overlaid to visualize these data. Differences between group means are determined with a one-way analysis of variance (ANOVA). The ODs of replicates (minimum of three) in each AgNP exposure group are natural log transformed, and the mean is calculated along with their 95% confidence interval (CI). These data are plotted verses time (ln OD vs. time) to create a growth curve. Growth of AgNP exposed cells are compared to the unexposed control.

4.1.1 Key results provided

The calculated doubling times, observable growth defects (e.g., increased lag-phase, lower final OD), and the MIC of the AgNP are the key results from this assay. As long as the same medium, cell number, and temperature are used, then samples from different experiments are directly comparable and can be analyzed together.

4.1.2 QA/QC considerations

It is important to accurately determine initial cell number in each assay as differences in cell number can influence the MIC and any observed growth defects. Additionally, the AgNP should be freely suspended or soluble in the medium without precipitation. The AgNP should also not significantly interfere with OD measurements (Petersen 2015; Petersen et al. 2015; Elliot et al. 2017). The degree to which the AgNP interferes with OD measurements of cell growth can vary with particle size, agglomeration, and concentration. Optical interference is defined as having an OD, such

that it prevents OD measurements of cell growth. It is recommended that when an AgNP OD > 0.1 or if the AgNP OD is not stable, growth monitoring by OD should not be used. In such cases, a MIC should be determined on a solid medium. It should be noted that it may be possible to lower the concentration of the AgNP such that the OD of the AgNP does not interfere with cell growth monitoring. Also, ODs other than 595 nm can be used (e.g., 530 nm, 600 nm, 630 nm) to allow cell growth monitoring. In these cases, a solid medium MIC assay only should be done at concentrations equal to, and above the interfering concentration of AgNPs. If these parameters are not met, an MIC can be determined on solid medium.

A main QA/QC consideration for this assay is sterility, whereby the negative controls containing only media, and media containing AgNPs, do not produce cell growth during the course of the assay. Sterility should be maintained by using aseptic technique during all aspects of bacterial culturing. It is recommended that a biological safety cabinet be used when available. Control (positive and negative) must be run on each plate to ensure that replicates and different NPs are directly comparable, as positive untreated controls should show consistent average doubling times and growth curves. Assays should be repeated if any negative controls or blanks show any signs of growth or if positive controls are disparate (Elliot et al. 2017).

4.2 Solid media MIC and MBC reporting

4.2.1 Analysis of results

Raw spot plate CFU count data are analyzed to determine CFU/ml for each spot. Cell number distributions are plotted in a box-and-whisker plot with the raw data points overlaid to visualize the data using data from at least three spots. Differences between group means are determined with a one-way ANOVA. Growth of AgNP exposed cells are compared to the unexposed control.

4.2.2 Key results provided

When an MIC is determined on a solid medium the key results are the establishment of an MIC concentration and observation of reduced cell numbers, indicating growth defects at the AgNP concentration being tested. As long as the same medium, cell number, incubation temperature,

and time are used, then samples from different experiments are directly comparable and can be analyzed together.

When an MBC is determined on a solid medium, the key results are the establishment of a MBC concentration and identification of the inhibitory mode of action. If cell growth is abolished in the MBC assay, the mode of action is bactericidal, whereas if cell growth is observed, the mode of action is bacteriostatic. As long as the same medium, cell number, incubation temperature, and time are used, then samples from different experiments are directly comparable and can be analyzed together.

4.2.3 QA/QC considerations

It is important to accurately determine the initial cell number in each assay, as differences in cell number can influence the MIC and/or MBC, and any observed growth defects. Additionally, the AgNP should be evenly dispersed in the solid medium (Petersen et al. 2014; Petersen et al. 2015). Spot plates should be dry before applying cells. These plates should be poured the day before and let dry overnight, or stored at 40 °C, and dry a minimum of one hour in a 37 °C incubator.

A main QA/QC consideration for these assays is sterility. Sterility should be maintained by using aseptic technique during all aspects of bacterial culturing. It is recommended that a biological safety cabinet be used when available. Control (positive and negative) must be run on each plate to ensure that replicates and different NPs are directly comparable, as positive untreated controls should show consistent average cell numbers after outgrowth. Assays should be repeated if any negative controls or blanks show any signs of growth, or if positive controls are disparate (Elliot et al. 2017).

4.3 Nanoparticle reporting

4.3.1 Analysis of results

The NP characterization is typically analyzed by the supplier, and should be reported. When these data are not available, it should be determined during toxicity studies.

4.3.2 Key results provided

For each NP tested, the particle purity, average size and size range, the type of coating, the delivery state (wet/dry), what dispersants were used, medium composition, and pH should be reported at a minimum. Storage conditions of the NP should also be reported.

4.3.3 QA/QC considerations

The media used can strongly and rapidly effect the properties, characteristics, and behaviors of NPs. If a dispersant, NP coating, or impurities are used, their cytotoxicity should be assessed so toxicity is not erroneously assigned to the AgNP (Petersen et al. 2014; Petersen et al. 2015; Petersen 2015). It is important to report the medium used (including composition and pH), and follow all manufacture's storage recommendations for AgNPs, typically 4 °C in the dark. It is recommended to complete the cytotoxicity testing as quickly as possible after acquiring the NP as prolonged storage has been known to influence NP properties (Petersen et al. 2014). If storage is prolonged, the NP characteristics should be confirmed to be unaltered.

5 Conclusion

It is increasingly important to understand the toxicity of nanomaterials with their rapidly expanding applications. Furthermore, the use of standardized methods allows comparisons between lab settings (e.g., exact NP, media used, strain tested, cell concentration used, etc.). This aspect is important when trying to adequately assess cytotoxicity data from multiple labs. Herein, is described a standardized method of analyzing the cytotoxicity of AgNPs on bacteria both in liquid and solid media. Furthermore, the mechanism of inhibitory action, if present, may be determined by MBC assays. These determinations can be quickly assessed in a matter of days using rapid culturing techniques and data analysis pipelines. This quick toxicity assessment will be advantageous for making informed decisions about products prior to their development for industrial and consumer applications. While this SOP primarily concerned AgNPs, it may be used or modified for use to assess bacterial cytotoxicity of other nanomaterials, expanding the utility of this SOP.

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14. ABSTRACT Materials at the nanoscale have physiochemical properties that differ from their bulk material. Consequently, the growing use of nanomaterials in industrial and consumer products raise important questions about their environmental fate. The potential cytotoxic effects on microbial communities is an important safety and material life cycle consideration, as microorganisms play a vital role in environmental nutrient cycling, influence health, and disease of plants and animals, including humans. This Scientific Operating Procedure (SOP) describes a set of methods to determine the cytotoxic effects of nanomaterials on bacteria. Initial growth effects, including minimal inhibitory concentration (MIC), can be monitored by a standardized microplate broth dilution assay when the nanomaterials stay suspended and do not inhibit optical density (OD) measurements. When these requirements are not met, a solid agar dilution plate MIC assay can be used whereby toxicity is determined by colony counts on agar plates. A standard minimum bactericidal concentration (MBC) assay is also detailed and can elucidated the mode of action of nanomaterials that have an MIC.					
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