Feasibility of using a standardized *Caenorhabditis elegans* toxicity test to assess nanomaterial toxicity

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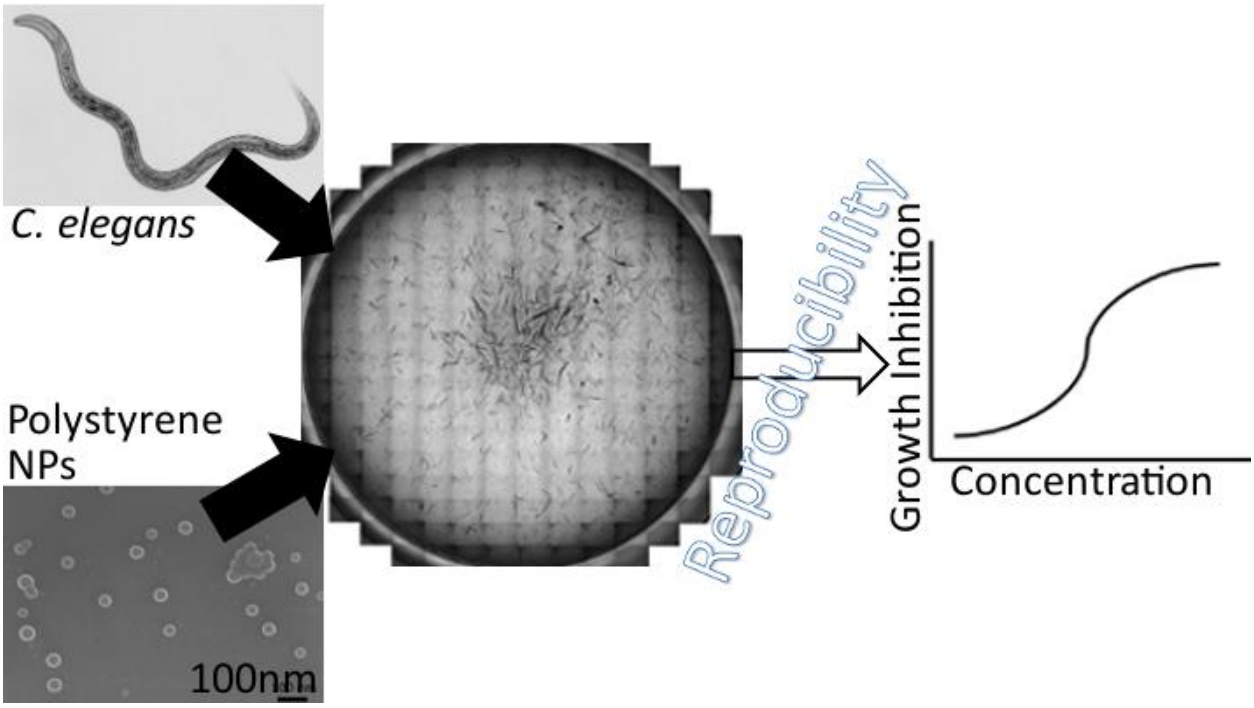

Abstract

Increasing production and use of engineered nanomaterials (ENMs) has generated widespread interest in measuring their environmental and human health effects. However, the lack of standardized methods for these measurements has often led to contradictory results. Our goal in this study was to examine the feasibility of using a standardized *Caenorhabditis elegans* growth and reproduction based toxicity test designed for use with dissolved chemicals to assess ENM toxicity. Sensitivity testing of seven key experimental factors identified by cause-and-effect analysis revealed that bacterial feed density and plate shaking had significant effects on growth inhibition by a reference toxicant, benzylcetyldimethylammonium chloride (BAC-C16). Bacterial density was inversely proportional to experimental EC_{50} values, while shaking the plates during the assay caused a substantial decrease in nematode growth and reproduction in control nematodes. Other factors such as bacterial viability, organism maintenance, and media

type showed minimal effect on the test method. Using this assay with positively charged polystyrene nanoparticles (PSNPs) revealed that the variability in the PSNP EC_{50} values was larger compared to those of BAC-C16. Additionally, while media type and bacterial viability did not impact BAC-C16 toxicity, PSNP toxicity differed substantially when these parameters were changed. PSNPs were more toxic in K^+ medium and S-basal compared to M9 and feeding nematodes with UV killed *E. coli* decreased toxicity of PSNPs. Test validity with ENMs and modifications that can be made to adapt the standard *C. elegans* toxicity assay for use with ENMs are discussed.

Introduction

Understanding the impact of increased production and use of engineered nanomaterials (ENMs) on the environment and human health is essential for sustainable commercialization of ENMs. Although researchers have been testing the hazards associated with ENMs for over a decade, [1-3] the lack of standardized methods and difficulties associated with ecotoxicity testing of ENMs has hindered these efforts and produced inconsistent results. [4, 5] For example, while Ag is a known biocide, the impact of size of Ag ENMs on toxicity is unclear [6] as are the mechanisms associated with the ENM toxicity. [7] A number of researchers attribute the toxic effects of Ag ENMs to dissolved Ag [8-10] while others show nanoparticle-specific effects. [11, 12] Differing results among laboratories may be attributed to many factors such as differences in protocols (i.e., ENM dispersion procedure, exposure duration), differences in ENMs of the same elemental composition (lot to lot manufacturer variability, size, surface coating, impurities), and improper controls leading to artifacts associated with ecotoxicity testing of the ENMs. [13] The choice of test organism and length of the assay also have a considerable impact on resulting toxicity of ENMs. Very high concentrations (> 500 mg l^{-1}) of carbon nanotubes (CNTs) had little to no effect on fish [14, 15] or amphibians, [16] yet much lower concentrations (< 10 mg l^{-1}) showed dose-dependent effects in copepods, [17] *Daphnia magna*, [18, 19] and marine mussels. [20] Additionally, CNT concentrations causing chronic *D. magna* toxicity [19, 21] are over an order of magnitude lower than those for short-term immobilization experiments. [22, 23] Chronic assays that measure sub-lethal endpoints, which are more sensitive to lower, more environmentally realistic concentrations, are needed to understand potential ecological impacts of ENMs. [24-26] These chronic assays show effects at orders of magnitude lower concentrations than acute assays. [27, 28]

The nematode *Caenorhabditis elegans* (*C. elegans*) is rapidly becoming a model for environmental and developmental toxicological research. [2, 29-33] Nematodes are possibly the most abundant multicellular organism on the planet and are important organisms in benthic and soil food webs. [34, 35] Therefore, using them as model organisms in toxicological assays would provide insight into the potential impacts of pollutants on the environment. Additionally,

C. elegans is well suited to toxicity assays due to its ability to grow and reproduce in soil and aqueous environments, ease of culture, and importance in soil food webs. In some cases, toxicity results with *C. elegans* have even correlated well to endpoints in mouse and rat studies, [36, 37] thus providing important data at a fraction of the effort and cost. *C. elegans* toxicity measurements can provide a strategy for chemical or ENM screening [2] to facilitate tiered testing approaches and minimize animal testing.

The International Standards Organization (ISO) published a standard toxicity assay with *C. elegans* [38] that allows the assessment of a potential toxin or contaminated environmental sample on sublethal physiological parameters (growth and reproduction). [39] The method is designed for use with dissolved chemicals or contaminated soils or sediments and guidance for using this method with ENMs is not readily available. This is important because ENMs may need to be dispersed prior to toxicity testing and may agglomerate, settle, and/or dissolve during the test and substantially change their toxicity. [1, 3, 40] Although the ISO method for *C. elegans* toxicity testing is available, it appears to only have been used for testing FeOx [41] and, with modification, for testing TiO_2 ENMs. [42] Other researchers have used a variety of non-standardized methods that included different media, exposure time, feed density, and endpoints.[43-46] Unsurprisingly, the studies yielded different findings and the variable conditions limit direct data comparison among laboratories. Even if the ISO standard method was used more often, researchers who use the ISO method for nanoecotoxicity testing may make different ENM-specific modifications, thus leading to additional variability in the results among different laboratories.

The purpose of this study was to assess the feasibility of using the *C. elegans* ISO method with ENMs by determining the impact of test modifications, that may be required for ENMs, on the toxicity results. In order to assess what steps of the ISO protocol may have the largest impact on the assay results, a cause-and-effect (C&E) analysis was performed. [47] This approach, which is a thought exercise originally developed for quality manufacturing and used for propagation of error analysis in analytical chemistry, identifies the expected major “causes” of variability and their “effect” on the assay result. This information was then used to conduct sensitivity testing of the ISO method by modifying various parameters of the protocol and determining how they impacted the toxicity results for a positive control. Our hypothesis was that this approach could provide information on the robustness of the test procedure and reveal which assay steps have the greatest impact on the variability of the results. These steps could then be modified to decrease variability or control measurements could be incorporated to more carefully track these sources of variability. After the sensitivity testing, a plate layout with multiple control measurements was developed and used to test the toxicity of a model ENM: positively charged polystyrene nanoparticles (PSNPs). PSNPs were selected because they could be purchased in a highly concentrated, stable suspension and their toxicity was hypothesized to be predominantly

attributable to the positively charged surface coating on the ENMs and not to dissolved ions or other non-specific nano effects. We then performed similar sensitivity testing with PSNPs to identify possible artifacts of the assay and evaluate the impacts of changing different parameters on the assay results with an ENM.

Methods

Overview of *C. elegans* toxicity assay

The duration of the ISO *C. elegans* assay is 96 h, during which time juvenile *C. elegans* mature into adult hermaphrodites, become gravid, lay eggs, and the eggs hatch into new juveniles. The test includes a bactericidal positive control, benzylcetyldimethylammonium chloride (BAC-C16), for which preliminary EC_{50} values for growth and reproduction have been established. The nematodes are fed *Escherichia coli* (OP 50 strain) in a defined bacterial density during the assay. Procedures for the toxicity assay were followed as detailed in ISO 10872 [38] and described in the supplementary information (Fig. S1). Briefly, 10 first stage juvenile (J1) *C. elegans* were added to each well of a 12 well plate along with the test material and *E. coli* as a food source. The plate was incubated at 20 °C for 96 h, at which point the nematodes were killed by heating and stained. In order to measure nematode length and to determine the number of new young nematodes, the plates were imaged using light microscopy. Quantitative assessment of growth and reproduction inhibition were determined based on nematodes in control wells without added toxicants.

Cause-and-effect analysis

A C&E analysis was conducted to identify which experimental factors may impact the test results by examining each step of the assay and determining how alterations of the protocol (that may be helpful for ENM stability) may impact results. The resulting output is a C&E diagram with main branches that depict steps that are major sources of variability and side branches that describe sources of variability that contribute to the main branches (Fig. 1 and Table S1). Information from this analysis was then used to design experiments that implemented sensitivity testing for quantifying the variability from the components of the C&E diagram.

Nematode cultures

Wild type, Bristol strain N2 *C. elegans* were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota) and maintained on nematode growth medium (NGM) with *E. coli* as feed. The nematodes were allowed to starve and become dauer larvae, which can survive for several months without feed. First stage juvenile (J1) *C. elegans* were obtained by

plating dauer larvae on a fresh *E. coli* lawn grown overnight on a NGM plate at 37°C and allowing the plate to incubate at 20 °C for 72 h.

Test material preparation

Polystyrene nanoparticles (PSNPs) were purchased from Bangs Laboratories (Bangs Laboratories Inc., Fishers, IN, USA) and were described as amine coated with a diameter of 58 nm. Primary particle size of 200 particles was measured via scanning electron microscopy using a Zeiss NVision 40 (Zeiss International, Oberkochen, Germany) focused ion beam/scanning electron microscope operating at 15 kV, and was determined to be 51 nm ± 9 nm (Fig. S2). To understand the behavior of ENMs in the test media across the duration of the study, a 100 mg l^{-1} suspension of PSNPs was prepared in 50 % S basal, K+ medium, and M9 (media composition described in SI) and the particle size was measured via dynamic light scattering (ZetaPALS Zeta Potential Analyzer, Brookhaven Instruments Corporation, Holtsville, NY) immediately and after the suspension was placed into a 20 °C incubator after 96 h. BAC-C16 (Acros Organics (97 % pure), Geel, Belgium) and PSNPs were prepared by diluting the samples in ultrapure water to produce concentrations twice as much as those used in the assay. The water was vortexed during addition of PSNPs to help maintain stability of the suspension. [48] Although the ISO standard suggests testing only a concentration of 15 mg l^{-1} of BAC-C16 for routine analysis, a range of concentrations from 3.5 mg l^{-1} to 40.5 mg l^{-1} were tested to produce an EC_{50} value during each experiment. To ensure that the PSNP coating or any other dissolved component that may have remained from synthesis was not causing a toxic effect, an 800 mg l^{-1} PSNP suspension was prepared in water, allowed to settle for 2 h, and then passed through a 0.02 µm filter. The resulting solution was used as an ENM filtrate control in the toxicity assay. [13, 22] In order to test all of the concentrations of BAC-C16 as well as PSNPs and controls associated with the PSNPs, a novel plate design was implemented as illustrated in Fig. S3.

E. coli preparation

A suspension of *E. coli* was prepared prior to the toxicity assay by inoculating 1 l of sterilized Luria Broth (Miller's LB broth base, Invitrogen, Thermo Fisher Scientific) with 100 µl of a frozen culture of *E. coli*. This culture was set on a shaker incubator at 37 °C and 15.7 rad s^{-1} (150 rpm) for 17 h. The culture was then transferred into 250 ml polypropylene centrifuge tubes and spun at 2000 x g (Allegra 25R Centrifuge, Beckman Coulter, Fullerton, CA, USA) and 4 °C for 20 min. The supernatant was decanted and the bacteria pellets resuspended into 50 ml of M9. This was repeated two more times. This suspension was then diluted in M9 and measured using a turbidity meter (HI 88713, Hanna Instruments, Woonsocket, RI, USA), which was calibrated using four formazin suspensions ranging from 15 formazin absorbance units (FAU) to 2000 FAU. [49] This calibration curve enabled the calculation of the dilution needed to obtain the specified *E. coli* concentration of 1000 FAU set forth in the ISO *C. elegans* protocol. [49] A 5 mg ml^{-1} solution of

cholesterol (NIST SRM 911c) dissolved in 100 % ethanol was then added to the feed suspension to achieve a 0.2 % v/v concentration of cholesterol.

Toxicity assay

The test wells were prepared by adding 500 µl of the test material and 500 µl of the *E. coli* suspension to each well. Two methods were used to obtain J1 nematodes for the toxicity tests, either a filtering method or a bleaching method. For the filtering method specified in ISO 10872, nematodes were washed from the culture plates onto a 5 µm polyester mesh filter (Hepfinger, Munich, Germany) using 8 ml of M9. The filtrate contained mainly J1 nematodes, however, second stage juveniles (J2) nematodes were also present. To avoid this, nematodes were also synchronized using a standard bleaching protocol adapted from [50] in which a mixed culture of nematodes were exposed to a sodium hypochlorite and sodium hydroxide solution for 10 minutes, washed with sterile water three times and the eggs were allowed to hatch in sterile water overnight. Bleached nematodes were only J1 stage as development is arrested with no food present. Ten J1 nematodes were added to each well and the test was initiated by placing the plates into a 20 °C incubator, in the dark, and leaving them undisturbed for 96 h. All J1 nematodes not used in the test were stained with Rose Bengal (500 µl of a 300 mg l^{-1} stock added to 5 ml), heated at 80 °C for 10 min to kill and straighten them, 30 individuals were measured, as described in the SI, to determine the initial nematode length. At the end of the test, 200 µl of a 300 mg l^{-1} stock of Rose Bengal was added to each well and the plate was heated at 80 °C for 10 min to kill and straighten all of the nematodes. The plate was allowed to cool for at least 1 h prior to imaging. All plates were stored at 4 °C and imaged within one week after the experiment concluded. Imaging details can be found in the SI. After imaging, total length of adult nematodes was measured and young were counted. Reproductive counts are expressed as young per adult hermaphrodite.

Sensitivity testing

To experimentally determine which of the six parameters identified in the C&E analysis (Table S1) had the greatest impact on the assay results, the identified parameters were adjusted and were compared to those obtained from the conditions indicated in the ISO standard. We tested effects of 1) the type of culture matrix from which nematodes were harvested (plate versus liquid culture), 2) two different manufacturers of BAC-C16 (Acros Organics (97 % pure), Geel, Belgium, was mainly used in the study, and compared to Alfa Aesar Ward Hill (95 % pure), MA, USA), 3) different assay media (S-basal medium, K^+ medium, and M9 medium), 4) the viability of the *E. coli* feed, 5) the size of wells (12 well and 24 well plate) 6) the feed density during the assay, and 7) whether the plates were shaken during the assay. Details of each test can be found in the SI.

198 Data analysis

199 Mean growth of nematodes in each well was calculated by subtracting the mean length of adult
200 hermaphrodites by the mean length of J1 nematodes measured at the start of the assay.
201 Inhibition of growth (G_I) was calculated for each nematode as follows:

202 $$G_I = 100 - \frac{L_F - L_I}{G} * 100$$

203 where L_F is final length of the individual nematode at the end of the assay, L_I is the mean initial
204 J1 length at the start of the assay, and G is the mean growth of the control nematodes during
205 the assay. Inhibition of reproduction (R_I) was calculated for each well as follows:

206 $$R_I = \frac{R_C - R_W}{R_C} * 100$$

207 where R_C is the mean reproduction per adult hermaphrodite found for the control wells and R_W
208 is the reproduction per adult hermaphrodite found in the test well. EC_{50} for growth and
209 reproduction was determined using a four parameter logistic function in GraphPad Prism (V
210 6.04, GraphPad Software, Inc).

211 **Results and Discussion**

212 C&E analysis

213 The C&E analysis provided a framework for identifying the factors of the assay that may cause
214 the greatest variability or uncertainty in the assay measurements. Six main categories were
215 identified: organism maintenance, the reference chemical, bacteria, the assay protocol,
216 microscopy, and ENM specific issues (Fig. 1 and Table S1). These branches include the
217 parameters outlined in the ISO protocol and potential modifications of the protocol for use with
218 ENMs. Branch 1 concerns the culturing procedure for *C. elegans*. The nematodes can be
219 cultured on agar plates containing a bacterial lawn or in liquid culture. The most common liquid
220 culture is S-basal media. However, nematodes cultured in liquid media are longer and thinner
221 than those from agar plates [51] and it is unknown if the culturing procedure impacts the
222 response of juveniles that are harvested for the exposure to toxicants. Branch 2 identifies
223 sources of variability related to the reference chemical, BAC-C16. BAC-C16, which is not easily
224 quantified, is difficult to dissolve in water, and little is known about its stability over time.
225 Therefore, there may be significant variability in the chemical toxicity among the batches from
226 suppliers and how well the chemical dissolves in water, all of which may impact *C. elegans*
227 growth inhibition results. Branch 3 concerns the *E. coli* density used in the assay, which is
228 difficult to measure accurately and could increase the assay variability as it may change during
229 the assay as a result of bacterial growth and interactions with the toxin. Branch 4 identifies

several factors in the assay protocol described in the ISO document, as well as adaptations for testing ENMs that may help keep the ENMs suspended. For example, different media preparations or incorporating plate shaking during the assay may impact the ENM suspension and the assay results. Branch 5 encompasses procedures for imaging nematodes for growth measurements. Sources of variability in this branch are associated with microscope calibration, nematode identification due to focus artifacts, interference from *E. coli* or debris, and user-to-user variability. Branch 6 catalogs ENM specific concerns included producing a reproducible dispersion, changes to the ENM during the assay (e.g., settling, dissolution), and toxicity of the ENM to the bacteria or interactions of ENM with bacteria such as heteroagglomeration.

Sensitivity testing with the BAC-C16 reference toxicant

The sensitivity testing of the ISO protocol yielded important insights regarding the parameters that impact the assay results (Fig. 2A-G and Fig. S4A-G). We found that the type of culture from which nematodes were harvested, the reference chemical vendor, the media that the assay was performed in, the *E. coli* viability, and the size of wells had minimal effect on the assay results with BAC-C16, as shown in Fig. 2A-E for growth, and Fig S4A-E for reproduction. The lack of impact on toxicity observed with BAC-C16 in different media may give researchers more flexibility to select which media works best for the ENM they are using. Media flexibility allows for use of more environmentally relevant media such as simulated porewater. [52] While the media we used in this study are some of the more commonly used in the literature, other media have shown differences in growth of nematodes. [53] Therefore, measuring control nematode growth in the chosen media is vital to understanding the potential impacts of a toxin. Our results suggest that the protocol described in the original ISO document is robust to media composition changes that were tested here. Changes in the nematode sensitivity to BAC-C16 was minimal when the nematodes were fed UV treated bacteria instead of live bacteria (Figure 2D and Fig. S4D). The EC_{50} for growth of BAC-C16 was 17.85 mg l^{-1} (95 % CI: 17.15 mg l^{-1} to 18.57 mg l^{-1}) for nematodes fed live bacteria and 14.87 mg l^{-1} (95 % CI: 14.17 mg l^{-1} to 15.61 mg l^{-1}) for nematodes fed UV treated bacteria. The EC50 for reproduction of BAC-C16 was 11.13 mg l^{-1} (95 % CI: 10.16 mg l^{-1} to 12.19 mg l^{-1}) for nematodes fed live bacteria and 10.33 mg l^{-1} (95 % CI: 0.06 mg l^{-1} to 1690 mg l^{-1}) for nematodes fed UV treated bacteria. Note the extremely high variability found for reproduction, especially for the nematodes fed UV treated bacteria. However, it is unclear if other researchers found impacts of bacterial viability on nematode growth. While we observed minimal impact of *E. coli* inactivation on growth of *C. elegans* similar to other studies, [46, 54] several studies have found that life span of *C. elegans* is increased when fed with growth inhibited or dead *E. coli*. [55, 56]

Of the parameters tested, changes in the *E. coli* feed density and plate shaking had the largest impact on assay results for BAC-C16. The feed density had a large impact on toxicity of BAC-

C16. At 100 FAU feed density levels, 15 mg l^{-1} of BAC-C16 completely inhibited nematode growth, while at 1100 FAU feed density, 15 mg l^{-1} of BAC-C16 did not affect growth (Fig. 2F). Similarly, nematodes did not reproduce below 500 FAU feed but reproduced as much if not more than the control at ≥ 900 FAU (Fig. S4F). This result indicates that the assay positive control is highly sensitive to feed density. Höss et. al. [57] found a similar result with Cd exposure and suggested that binding of Cd to *E. coli* cells may impact bioavailability of the metal. However, the method by which researchers measure bacterial density may impact the amount of feed being administered. Bacterial density measurements were conducted in this study using a turbidity meter as described in ISO method 10872, [38] but researchers use different methods to quantify bacterial density such as photometers, [47, 51] plate readers, [52] or simply specify a wavelength with no indication of instrumentation. [35, 37, 53] The impact of using different instruments to quantify bacteria densities is unclear, but our sensitivity testing suggests that a 50 FAU difference in bacterial concentration can change growth inhibition of BAC-C16 by as much as 19 % (Fig. 2F). It is unclear if this is due to the nematodes having access to different amounts of feed or if increasing amounts of *E. coli* decreases the availability of BAC-C16 to the nematodes. Distinguishing the direct toxic effect of a chemical on growth inhibition from an indirect effect on bacterial concentration is not possible with the current ISO method.

Shaking plates during the assay decreased control growth by >300 µm (approximately 19 % decrease) after 96 h and increased inhibition of growth of 15 mg l^{-1} BAC-C16 by >36 % compared to not shaking the plates (Fig. 2G). Similarly, shaking plates reduced reproductive output by approximately 70% compared to not shaking (Fig. S4G). Shaking the plates greatly increased test variability; the mean EC_{50} (± 1 SD) for growth with plate shaking was 20.5 ± 13.1 mg l^{-1} (n=16), yet decreased to 18.7 ± 2.6 mg l^{-1} (n=16) without shaking. While leaving the plates undisturbed during the assay may allow nematodes easier access to settled *E. coli*, aggregated ENMs may also settle on the bottom of the wells, potentially increasing exposure of the nematodes to the ENMs as well as changing exposure from ENMs to aggregates of these ENMs [58]. It is important to consider these features of the test system when interpreting the results of the assay with test ENMs. For example, choosing a media that minimizes aggregation, if possible, would help to alleviate this issue.

Sensitivity testing with PSNPs and comparison to reference chemical results

Based on the results of the C&E and sensitivity testing with BAC-C16, we designed the layout of 12 well plates that provide five control features to assess the quality of the results (Fig. S3). There are multiple advantages of testing the EC_{50} value of the reference chemical and ENM on each of three plates including that the plate-to-plate variability can be quantified and that the EC_{50} values for the BAC-C16 need to be within benchmark specifications for the ENM result to be considered valid. For our laboratory, the mean EC_{50} (± 1 SD) of BAC-C16 was 18.7 ± 2.6 mg l^{-1}

(n = 16) and ranged from 14.4 mg l^{-1} to 22.3 mg l^{-1} (Fig. 3A). Mean inhibition of growth at 15 mg l^{-1} BAC-C16 was 34.1 ± 12.5 % and ranged from 18.1 to 58.7 % (Fig. 3B). While our growth inhibition results were mainly within the 20 % to 80 % requirement as stated in ISO 10872, [38] several of our tests showed < 20 % inhibition at 15 mg l^{-1} BAC-C16. However, an interlaboratory study among eight laboratories showed a range of EC_{50} values for growth from 11.9 to 18.9 mg l^{-1}, [39] suggesting that our variability is similar to those in the interlaboratory study. This chemical control and specification can be used to qualify the robustness of the measurement process.

The results from conducting the assay with PSNPs three separate times (Fig. 4A) indicated that the mean EC_{50} for growth was 71.7 ± 37.2 mg l^{-1} and ranged from 42.7 mg l^{-1} to 113.7 mg l^{-1}. The mean EC_{50} for reproduction was 21.4 ± 10.5 mg l^{-1} and ranged from 10.0 mg l^{-1} to 30.7 mg l^{-1}. While no published research has investigated the toxicity of PSNPs on *C. elegans*, cellular toxicity assays indicate almost an order of magnitude lower EC_{50} values than those observed for *C. elegans*. [47] Several concentrations of BAC-C16 were tested in the same plates as the PSNPs to compare the variability between the two substances (Fig. 4B). The coefficient of variations of the growth EC_{50} values for three independent assays were 9 % and 52 % for the BAC-16 and PSNPs, respectively, indicating that the EC_{50} values were substantially more variable for PSNPs. No inhibition of growth was observed in the ENM filtrate control, suggesting that no leaching of toxic chemicals from the ENM occurred. However, there were differences in *E. coli* agglomeration in the presence of PSNPs and large *E. coli* agglomerates formed almost immediately after addition (Fig. S5B). This observation suggests an additional indirect toxicity mechanism (i.e., heteroagglomeration) that should be considered when testing ENMs. It is not clear if the observed toxicity is due to the ENM or due to a secondary effect that results from the ENM interaction with *E.coli* feed. For example, it is possible that the aggregates may change the availability of feed to the nematodes. Experiments to further dissect the observed nematode toxicity will be explored in a subsequent study.

Unlike results for BAC-C16, media composition had a strong influence on the toxicity of PSNPs (Fig. 5A and Fig. S6A), suggesting that care must be taken to understand the behaviors of the ENM in the system. Growth EC_{50} values for the three media were 23.7 mg l^{-1} (95 % CI: 21.4 mg l^{-1} to 26.2 mg l^{-1}), 5.9 mg l^{-1} (95 % CI: 5.5 mg l^{-1} to 6.3 mg l^{-1}), and 8.8 mg l^{-1} (95 % CI: 8.1 mg l^{-1} to 9.5 mg l^{-1}) for M9, K^+ medium, and S-basal respectively. For reproduction, EC_{50} values could not be calculated for M9 due to high variability but were 2.7 mg l^{-1} (95 % CI: 2.4 mg l^{-1} to 3.1 mg l^{-1}), and 2.8 mg l^{-1} (95 % CI: 2.7 mg l^{-1} to 3.0 mg l^{-1}) for K^+ medium and S-basal respectively; while these experiments were repeated at least twice, conducting the experiments with a different concentration test range may have enabled the calculation of reproduction EC_{50} values but was not tested. While K^+ medium has the lowest ionic strength and S-basal had the highest ionic strength of the three media we tested, PSNPs were least toxic in M9. This may be due to the fact that K^+ medium and S-basal contain two different types of divalent cations, Ca^{2+} and Mg^{2+},

while M9 contains only Mg^{2+}. As previously reported, the presence of divalent cations can potentiate ENM aggregation in liquid media, [59, 60] which may impact toxicity. Immediately after addition, PSNPs agglomerated in S-basal (mean ± SD: 1117.8 nm ± 15.2 nm) and M9 (199.4 nm ± 4.0 nm) but not in K^+ medium (64.2 nm ± 0.5 nm). After 96 h PSNPs increased in size in S-basal (1966.3 nm ± 512.3 nm) and M9 (649.6 nm ± 14.0 nm) but remained similar in K^+ medium (58.4 nm ± 0.5 nm). However, these measurements were run without E. coli present, the presence of which may impact PSNP agglomeration. The media composition may be highly relevant for other ENMs such as Ag ENMs which react readily with chloride; [61] a media without chloride salts may be needed to obtain the lowest EC_{50} values for Ag ENMs but such a media would have lower environmental relevance. [52] Similarly, bacteria viability influenced PSNP toxicity (Fig. 5B and Fig. S6B), but did not impact the toxicity of BAC-C16. EC_{50} values for growth were 38.1 mg l^{-1} (95% CI: 30.5 mg l^{-1} to 47.6 mg l^{-1}) for nematodes fed live E. coli and 45.4 mg l^{-1} (95% CI: 32.5 mg l^{-1} to 63.4 mg l^{-1}) for those fed UV killed E. coli. EC_{50} values for reproduction could not be calculated due to high variability. UV killed bacteria decreased PSNP toxicity, suggesting that the interaction between PSNPs and *E. coli* may be hindered when bacteria are UV killed. This suggests a potential assay modification to avoid this artifact. Similar to that of BAC-C16 results, feed density greatly impacted toxicity of PSNPs (Fig. 5C and Fig. S6C). At 70 mg l^{-1} PSNPs nematode growth was similar to that of the control when feed was increased to 1100 FAU but minimal to no growth was observed for feed densities between 100 and 550 FAU. Similarly, no reproduction was observed at 70 mg l^{-1} PSNPs until feed was increased to 900 FAU and at 1100 FAU, reproduction was similar to that of the control (Fig. S6C). Variability for all PSNP assays was increased compared to data for BAC-C16.

Conclusion

This paper describes a process to assess the robustness and reproducibility of an ISO *C. elegans* ecotoxicity assay and the utility of this assay for testing the potential effects of ENMs. Our cause-and-effect analysis followed by a sensitivity testing revealed that *E. coli* concentration and plate shaking have a large impact on nematode growth and toxicity of the control toxicant BAC-C16. Lastly, we found that *E. coli* concentration, bacterial viability, and media composition impacted PSNPs toxicity to *C. elegans*, illustrating the need to understand how ENM toxicity is impacted by assay parameters. Given that studies in the literature often use a range of *E. coli* concentrations and media compositions, the impact of these parameters should be better understood using a broader range of conditions (bacteria concentrations, media, and types of nanoparticles) to elucidate how data from multiple studies can be combined for environmental risk assessment. In addition, the development of a more precise and robust method for quantifying the bacteria concentration could help decrease the variability of the assay.

While our findings with PSNPs illustrate the need to better understand the main factors contributing to variability in assays when including ENMs, further experiments are needed to better understand the robustness of the assay for use with varying nanoparticles (e.g., with different surface coatings or sizes), because there may be biases or artifacts in the assay that were not uncovered by testing only a single nanoparticle. Based on the findings of this study, our recommendation is for this standardized method to be used more broadly in the nanotoxicology literature. If modifications are made to the assay for which the assay is sensitive (e.g., lower bacteria concentrations of different test media), it would be helpful to enable data comparability among laboratories to also test the ENP using the conditions described in the ISO assay. However, additional testing of the robustness of this assay with different ENPs may reveal other important biases or limits to the applicability of this assay which should also be taken into consideration.

The use of quality tools such as the cause-and-effect diagram and sensitivity testing allowed us to systematically identify the parameters of the nematode culturing and toxicity assay that had the greatest impact on assay results. This process can aid in reducing variability and increasing reliability of standardized ecotoxicity tests and other key environmental measurements.

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Certain commercial products or equipment are described in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that it is necessarily the best available for the purpose.

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505 Figure Captions

506 Figure 1. Cause-and-effect analysis of ISO 10872 protocol. The six main branches indicate the
507 factors that we have identified that have the greatest potential to cause variability in assay
508 results. For detailed descriptions see Table S1.

509 Figure 2. Sensitivity testing of ISO 10872 conducted by altering test conditions (shown in Figure
510 1) and comparing the outcome to the original protocol. The test parameters altered were (A)
511 the culture from which the nematodes were harvested for the assay, (B) the manufacturer of
512 the positive control BAC C16, (C) the media that the test was performed in, (D) bacterial
513 viability, (E) the assay performed in a 24 well plate instead of 12 well (F) the amount of feed
514 used in the assay (all exposures include 15 mg l^{-1} BAC-C16), and (G) whether the plates were
515 shaken or left undisturbed. For each plot, growth data shown are mean ± one standard
516 deviation, n=3 for each data point.

517 Figure 3. Control charting of EC_{50} values (A) and inhibition of growth at 15 mg l^{-1} (B) of *C.*
518 *elegans* exposed to BAC-C16 in 96 h standard toxicity assays conducted over several months
519 based on ISO 10872. Data presented as mean ± one standard deviation. The vertical bars
520 represent the date we stopped shaking plates during the assays.

521 Figure 4. Variability of the adapted toxicity assay for growth inhibition of A) PSNPs and B) BAC-
522 C16 conducted on three different days. Data are shown as mean ± one standard deviation. N =
523 3 wells, each with 10 nematodes.

524 Figure 5. Sensitivity testing of ISO 10872 containing PSNPs. The test parameters altered were
525 (A) The feed density, (B) the media that the test was performed in, and (C) the bacterial density.
526 Growth data presented as mean ± one standard deviation. For each experiment N = 3 wells,
527 each with 10 nematodes. Experiments were performed twice and data are combined.

Figure 1.

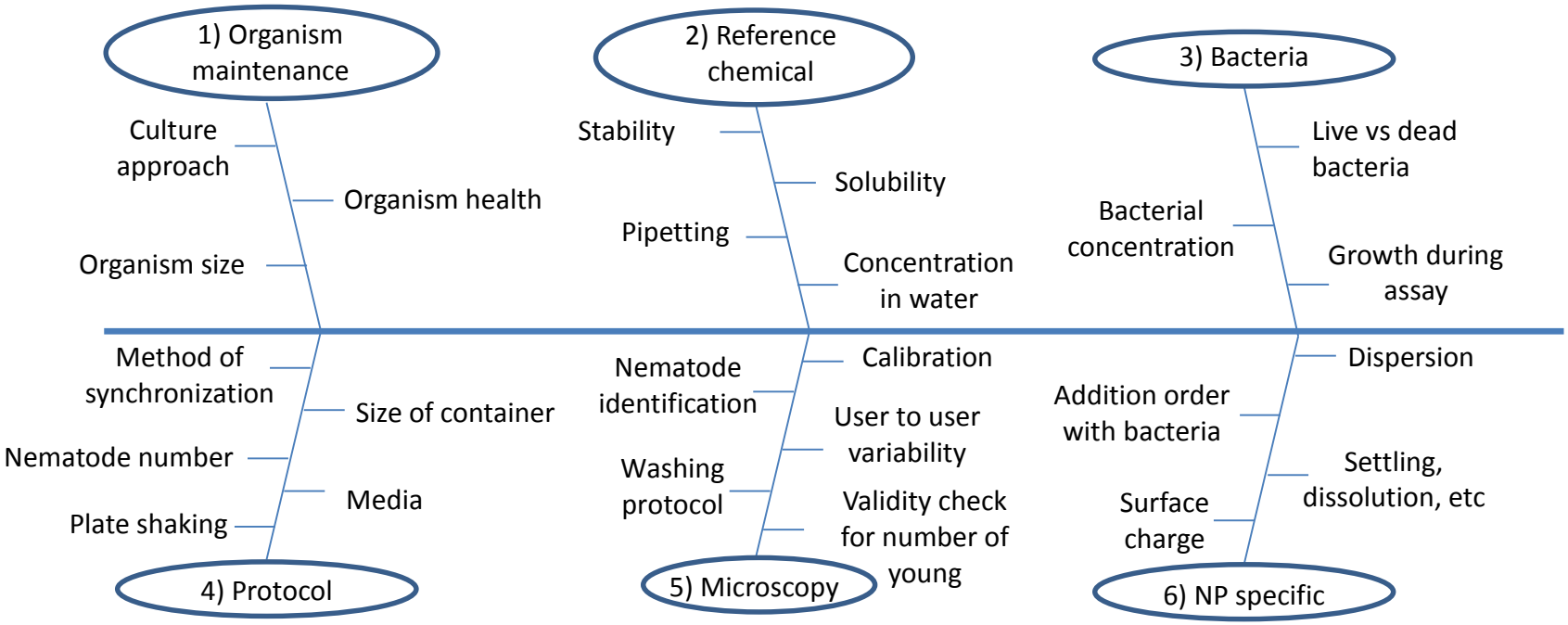

1) Organism maintenance
Culture approach
Organism health
Organism size
Method of synchronization
Size of container
Nematode number
Media
Plate shaking
4) Protocol
2) Reference chemical
Stability
Solubility
Pipetting
Concentration in water
Nematode identification
Calibration
User to user variability
Washing protocol
Validity check for number of young
5) Microscopy
3) Bacteria
Live vs dead bacteria
Bacterial concentration
Growth during assay
Dispersion
Addition order with bacteria
Settling, dissolution, etc
Surface charge
6) NP specific

Figure 2.

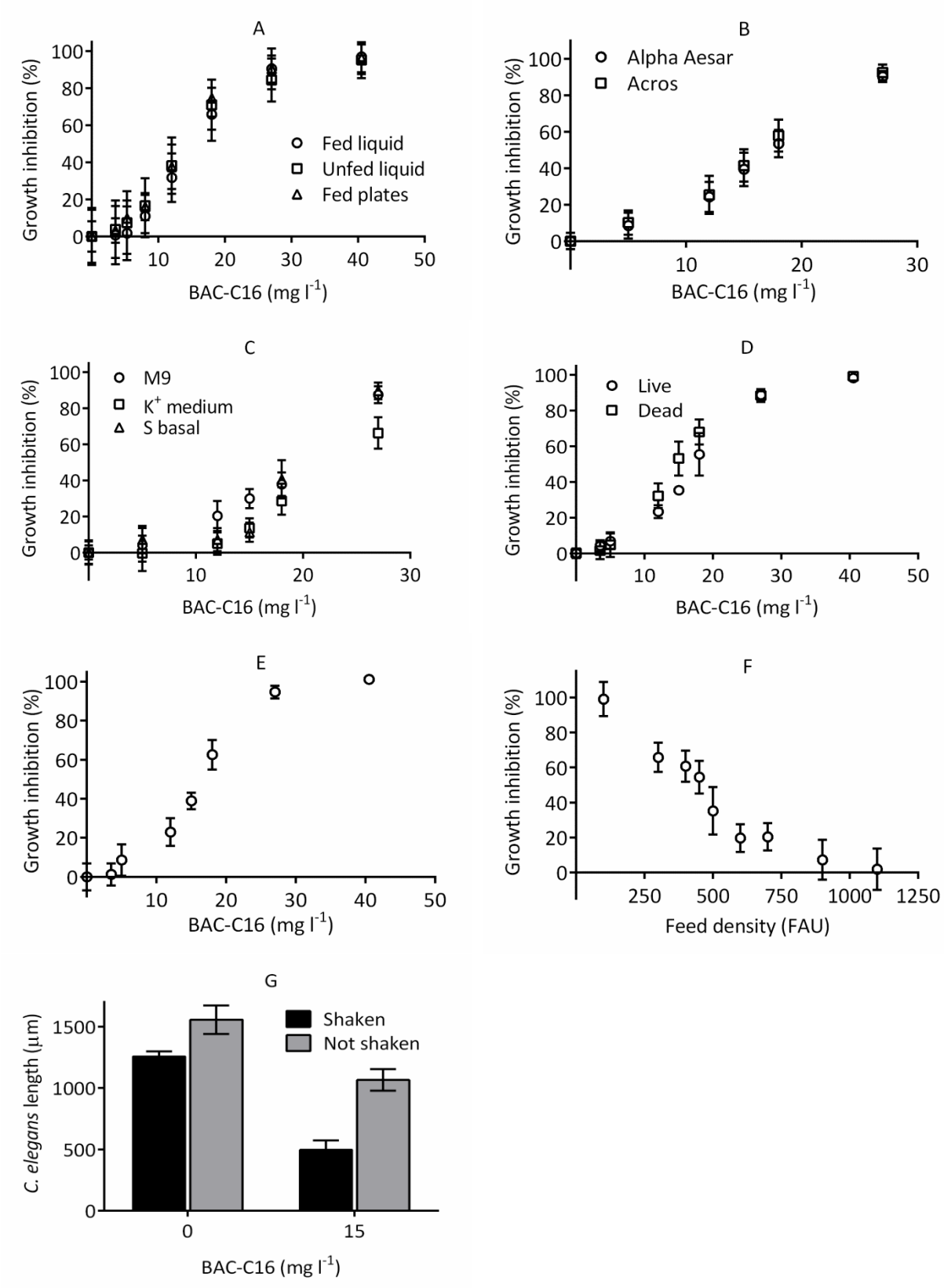

A
Growth inhibition (%)
BAC-C16 (mg l-1)
Fed liquid
Unfed liquid
Fed plates
B
Alpha Aesar
Acros
C
M9
K+ medium
S basal
D
Live
Dead
E
F
Feed density (FAU)
G
C. elegans length (µm)
Shaken
Not shaken

Figure 3.

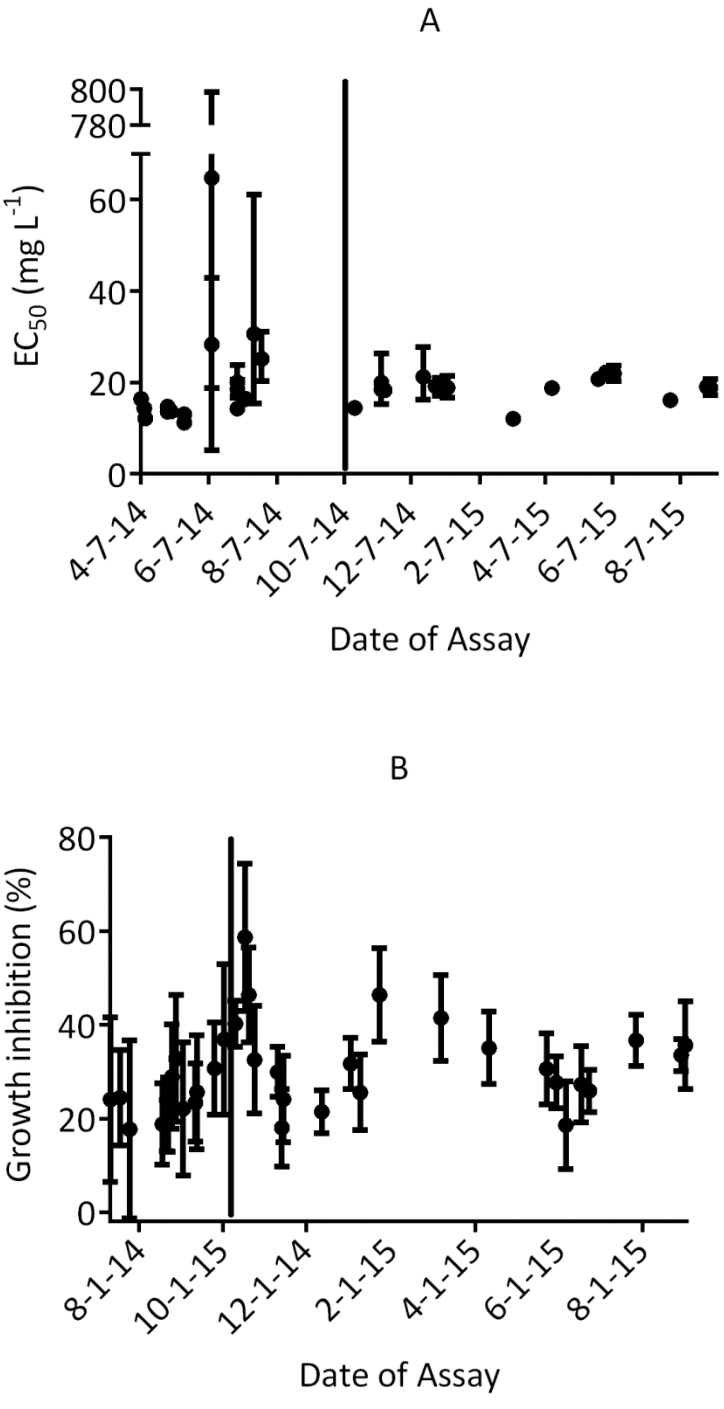

A
800
780
60
40
20
0
EC50 (mg L-1)
4-7-14
6-7-14
8-7-14
10-7-14
12-7-14
2-7-15
4-7-15
6-7-15
8-7-15
Date of Assay
B
80
60
40
20
0
Growth inhibition (%)
8-1-14
10-1-15
12-1-14
2-1-15
4-1-15
6-1-15
8-1-15
Date of Assay

Figure 4.

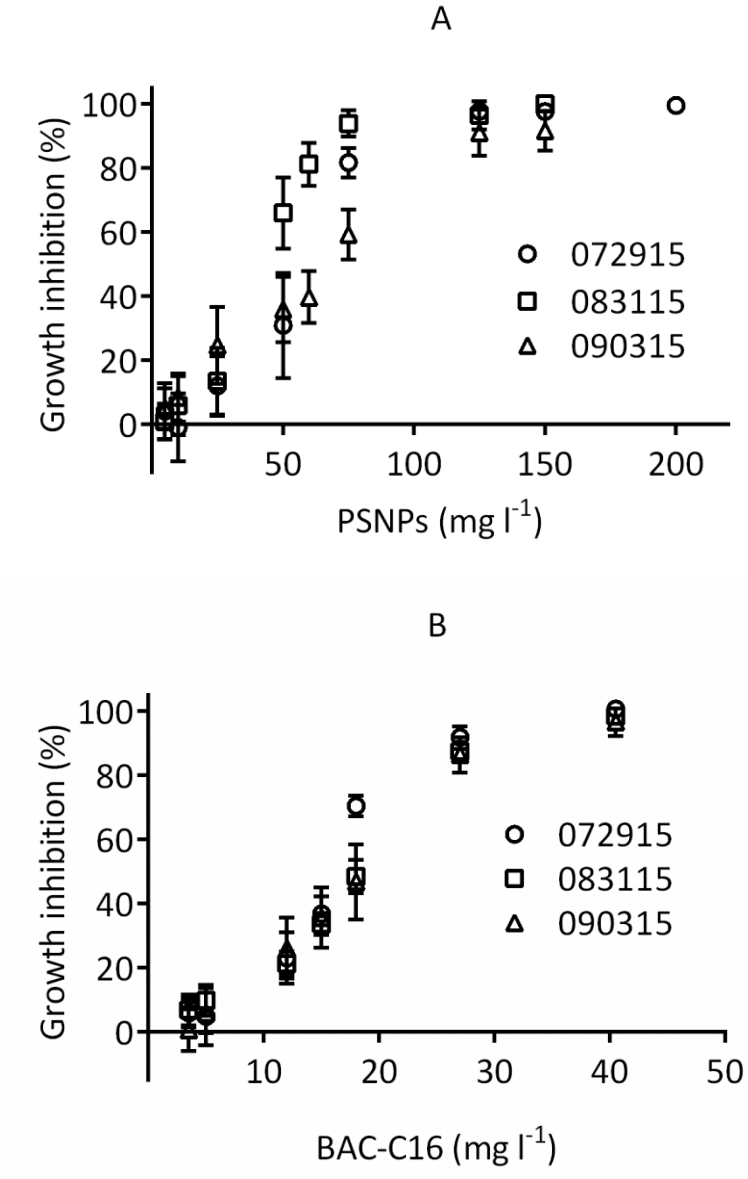
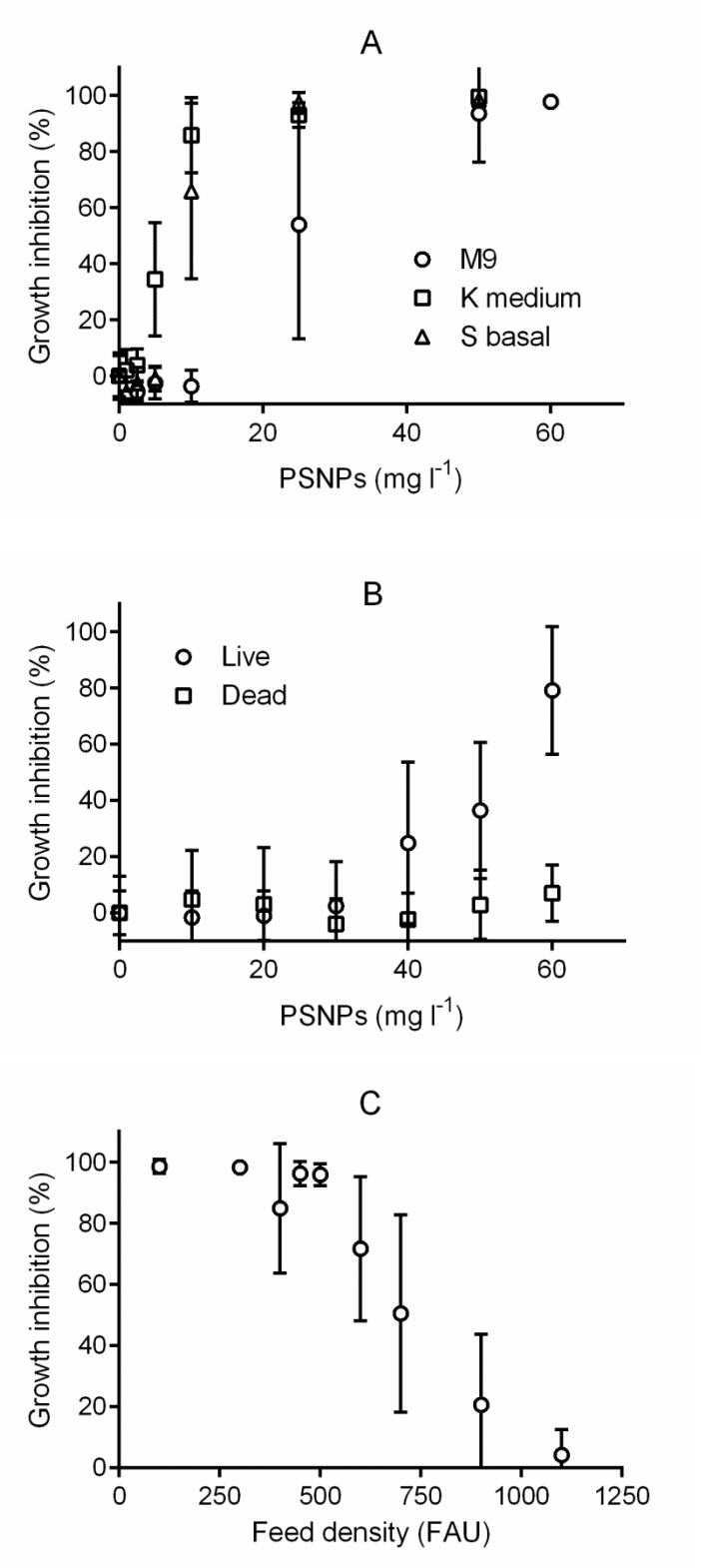

A
Growth inhibition (%)
100
80
60
40
20
0
50
100
150
200
PSNPs (mg l-1)
072915
083115
090315
B
Growth inhibition (%)
100
80
60
40
20
0
10
20
30
40
50
BAC-C16 (mg l-1)
072915
083115
090315

Figure 5.


A
Growth inhibition (%)
100
80
60
40
20
0
0
20
40
60
PSNPs (mg l-1)
M9
K medium
S basal
B
Growth inhibition (%)
100
80
60
40
20
0
0
20
40
60
PSNPs (mg l-1)
Live
Dead
C
Growth inhibition (%)
100
80
60
40
20
0
0
250
500
750
1000
1250
Feed density (FAU)