NNI Public Webinar

Practical Applications of 15 Years of NanoEHS Research: Measurements of Potential Ecotoxicological Risk

Speaker

Dr. Elijah Petersen
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Moderator

Dr. Treye Thomas
Program Manager
Chemicals, Nanotechnology and Emerging Materials Program Area, Consumer Product Safety Commission

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>> Treye Thomas: Good afternoon, everyone. Welcome to the webinar. We thank you for joining us today. My name is Treye Thomas, I'm a program manager for the Chemicals, Nanotechnology and Emerging Materials program at the U.S. Consumer Product Safety Commission. I also have the pleasure of serving as the National Nanotechnology Initiative's Coordinator for Environmental, Health, and Safety (EHS) research.

Today's webinar continues the 2019 NanoEHS webinar series. This year's NanoEHS webinar series highlights the considerable research progress in understanding the potential environmental and human health effects of engineered nanomaterials, or ENMs, since the NNI was authorized back in 2003.

This theme sets the stage for examining the progress towards meeting the NNI research strategy goals set out almost ten years ago, in 2011.
It's my pleasure to welcome and introduce our speaker, Dr. Elijah Petersen.

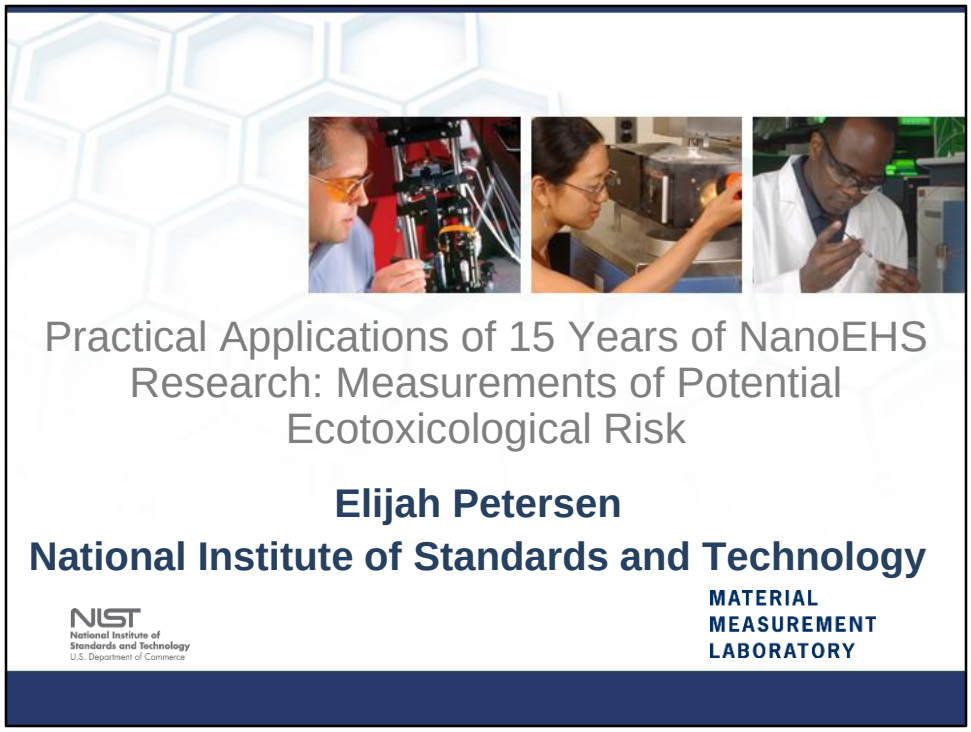
DR. ELIJAH PETERSEN

PhD in Environmental Engineering at University of Michigan
Fulbright Scholarship for Postdoc in Finland (2007)
Joined NIST as NRC Postdoc in 2009
Staff Scientist at NIST in 2010-present
2016 Sustainable Nanotechnology Organizing (SNO) Emerging Investigator Award
2018 SNO conference chair
US-EU Bridging NanoEHS Research Efforts, Ecotoxicology Community of Research US co-chair (2013-2018)
Associate Editor Nanotoxicology (since 2018) and Nanoimpact (since 2015)
Published over 70 papers

>> Treye Thomas: Dr. Petersen received his Ph.D. in environmental engineering at the University of Michigan. He was also Fulbright scholar and joined NIST as an NRC postdoc back in 2009. He currently serves as a Staff Scientist in the Biosystems and Biomaterials Division for NIST. His presentation will provide examples of the practical applications of progress in understanding and measuring engineered nanomaterials in the environment. Dr. Petersen's case study will also underscore the role of international collaboration and developing best practices, and in sharing information, to tackle these challenges.

We have budgeted time after the presentation for questions and answers. So please, as you are reviewing the slides and the presentation, have questions prepared for the end. Before I turn it over to Elijah, I do want to remind everyone that the NNI public webinar series is on nano.gov. You can find the NNI EHS research strategy on nano.gov as well. You can also follow us on Twitter, @NNInanonews.

So with that I will turn it over to you, Elijah.



>> Elijah Petersen: Thank you, Treye, for that introduction; and thank you to the NNCO for this invitation and the ability to share some of what NIST is involved in, in terms of developing robust methods for the potential environmental risks of nanotechnology.

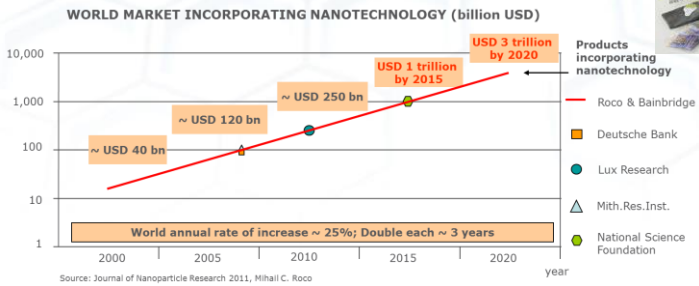
During this talk I'll mainly focus on some of the ongoing work at NIST on this topic, but there's a ton of work I will highlight at some point from a number of other universities and institutes. So there's been a lot done, and I will focus on a small subsection of it.

I think that, compared to when NNI started back in 2003, the science has dramatically improved since then.

Nanotechnology

Nanotechnology is on the rise

More than 1,500 nanomaterial containing consumer products on the market.



Key research needs

- Assessing potential environmental, health and safety risks require robust, reproducible methods
- Often toxicity assays needs modifications for use with nanomaterials but the impact of these changes on the assay results is unclear

→ Need robust toxicological assays

→ Need reliable analytical methodologies for ENM characterization.

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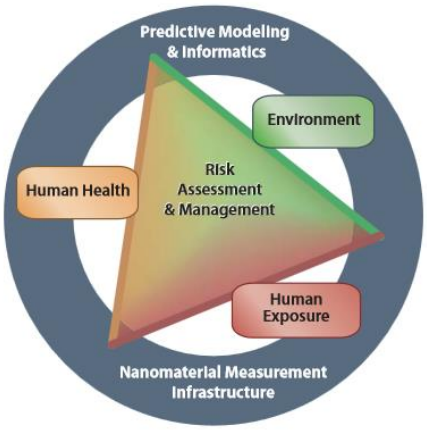
>> Elijah Petersen: So to give a little bit of background about nanotechnology, there's a lot of nanomaterial-containing consumer products on the market; there's predictions for more and more to be incorporated in future years. And with this we need to have good methods. They need to be robust and reproducible. We need to understand potential environmental health and safety risks.

And to do this we can, at times, leverage methods that have been developed previously for dissolved chemicals, but there may be a need to make changes to those assays for them to be robust for use with nanoparticles and avoid potential artifacts or biases. These robust toxicological assays are critical and another key part is the analytical methods for the engineered nanomaterial characterization during the experiments.

NIST ROLE IN NANOTECHNOLOGY ENVIRONMENTAL HEALTH AND SAFETY (NANOEHS)



National Nanotechnology Initiative 2011 Environmental Health and Safety Research Strategy

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>> Elijah Petersen: This picture comes from the 2011 Environmental, Health, and Safety Research Strategy from the NNI (see https://www.nano.gov/node/681). And in the center, you will notice that there is risk assessment and management. That's for the regulatory agencies to conduct. NIST is not regulatory agency. That is not our purview, but we do try to support this. But to do accurate risk assessment and management that's scientifically sound, we need to have measurements of environmental fate and effects, potential human exposure, and potential human health risks.

Underpinning that is predictive modeling and informatics, and what NIST was tasked with was the nanomaterial measurement infrastructure. This includes reference materials, documentary standards, and a number of other things, which I will touch on to some degree today.

NIST Reference Nanomaterials

Vincent Hackley

Gold nanoparticles (10, 30, and 60 nm)
Single-wall carbon nanotube (raw soot) and dispersed into three length populations
Titanium dioxide nanoparticles (made from Degussa P25)
2 nm silicon nanoparticles
Silver nanoparticles (75 nm, 10 nm in preparation)
Multiwall carbon nanotube (raw soot)

Can be useful for interlaboratory comparisons, instrument validation and calibration, and positive and negative controls for nanotoxicity studies

Critical for establishing comparability of nano-related measurements.

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>> Elijah Petersen: One of the things NIST is known for is our reference materials. Vince Hackley has been a real leader of this at NIST and was the person driving a number of these reference materials. Rob MacCuspie and his presentation, not the last one but the one before that, talked a lot about this as well. What's really valuable for this is it can support establishing comparability among nano-related measurements.

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Documentary standards

NIST participates in standards organizations that provide validated documentary standards on a range of topics

- Nanoparticle characterization using a range of instruments for all nanoparticles (DLS, TEM, etc.) through the NIST/NCL protocols
- Sonication protocols that provide reproducible, traceable NP sonication between instruments and laboratories
- MTS assay for cell toxicity from nanomaterials
- Guidance document for aquatic toxicity testing of nanomaterials

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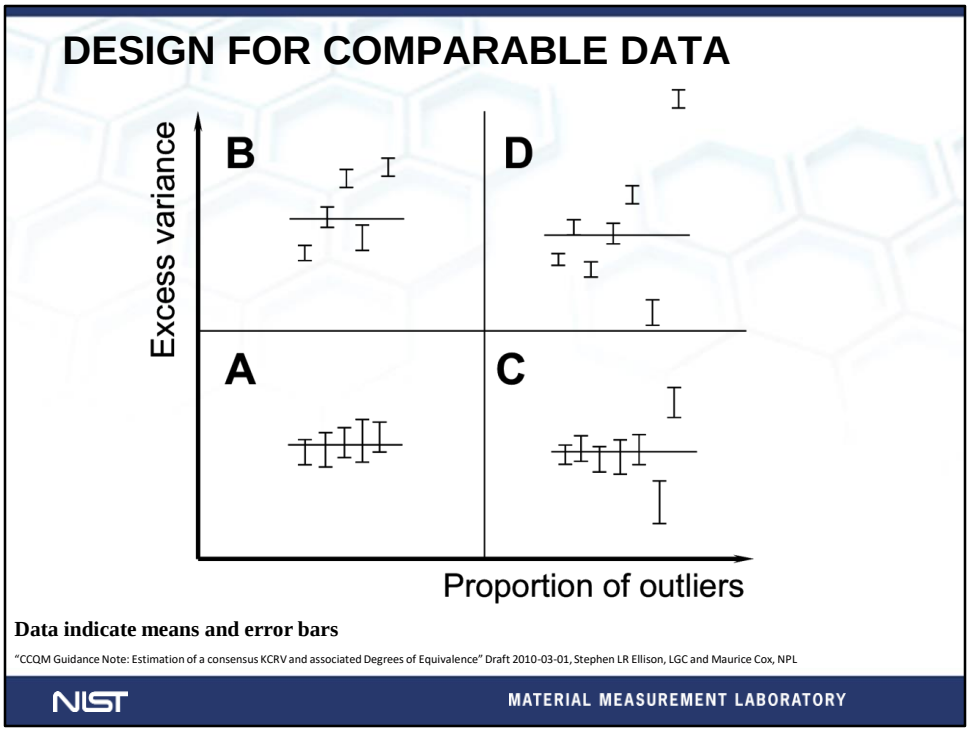
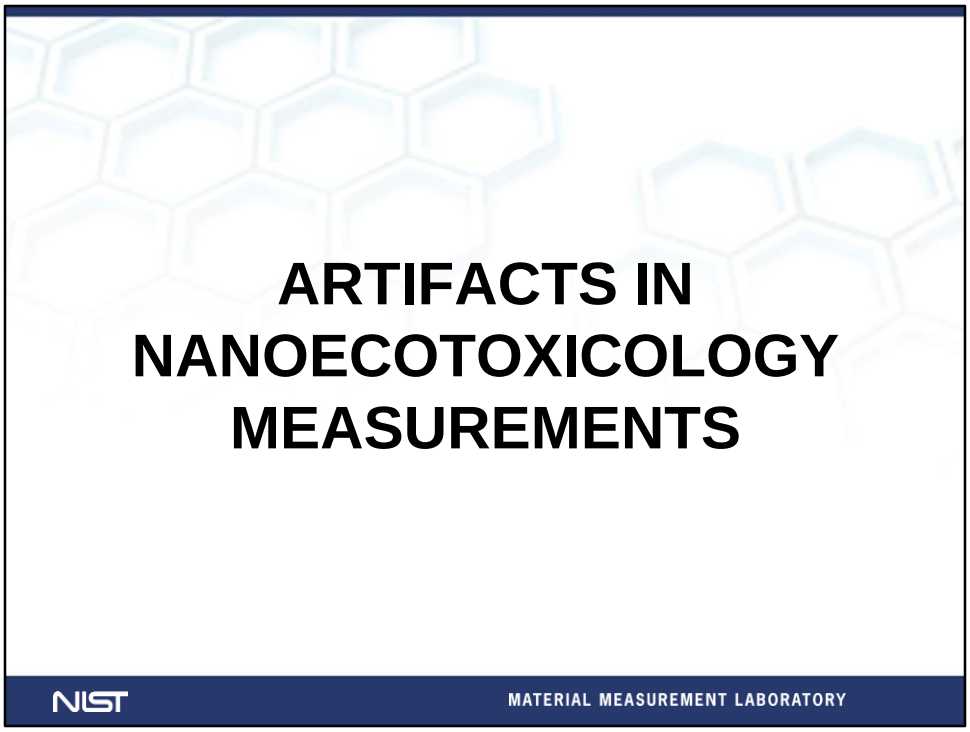
>> Elijah Petersen: At NIST, we are also highly involved in a number of documentary standards organizations, and efforts: ASTM E56, ISO TC 229, and the OECD Working Party on Manufactured Nanomaterials. And we have been involved for a long time in a number of different standards. We will mainly be focusing today on the last documentary standard that I've listed here, the guidance document for aquatic toxicity testing of nanomaterials.



>> Elijah Petersen: Our big focus at NIST, or one of them at least, is enabling comparable data. What we want to have is results among different laboratories for the same measurement similar to those on the bottom left quadrant, quadrant A. There's a minimal proportion of outliers and there's minimal variance. But how exactly do we do that? What you need is methods that are transferable, robust, and where you can have confidence that when you are conducting the assay, the results you are getting are reliable, valuable, and trustworthy.

This is especially important in terms of looking at potential environmental, health, and safety risks because if a company is developing a product, they may rely on a contract testing laboratory. Those laboratories would then be utilizing documentary standards that have been published and gone through a consensus process. The key is how can we have standards that are written in a way that these different contract testing laboratories can perform them and have results that are in good agreement with each other; that will enable commerce among different countries, and help get really good data while minimizing costs for companies producing nanotechnology-enabled products, having to make these type of measurements?



>> Elijah Petersen: One of the challenges is having comparable data, as there's a potential for artifacts or biases in these nanoecotoxicology measurements.

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Identification and Avoidance of Potential Artifacts and Misinterpretations in Nanomaterial Ecotoxicity Measurements

Elijah J. Petersen,[†,*] Theodore B. Henry,[‡,§,∥] Jian Zhao,[⊥] Robert I. MacCuspie,[#,∇] Teresa L. Kirschling,[○] Marina A. Dobrovolskaia,[◆] Vincent Hackley,[#] Baoshan Xing,[⊥] and Jason C. White[¶]

Artifacts can potentially occur at each step of nanoecotoxicology testing

1. Procurement of NPs (impurities, incorrect sizes)
2. Storage (dissolution, release of coatings)
3. Dispersion (ROS from ultrasonication)
4. Measurement of toxic endpoints (interaction with test reagents)
5. Characterization in tissues (misidentification using TEM)

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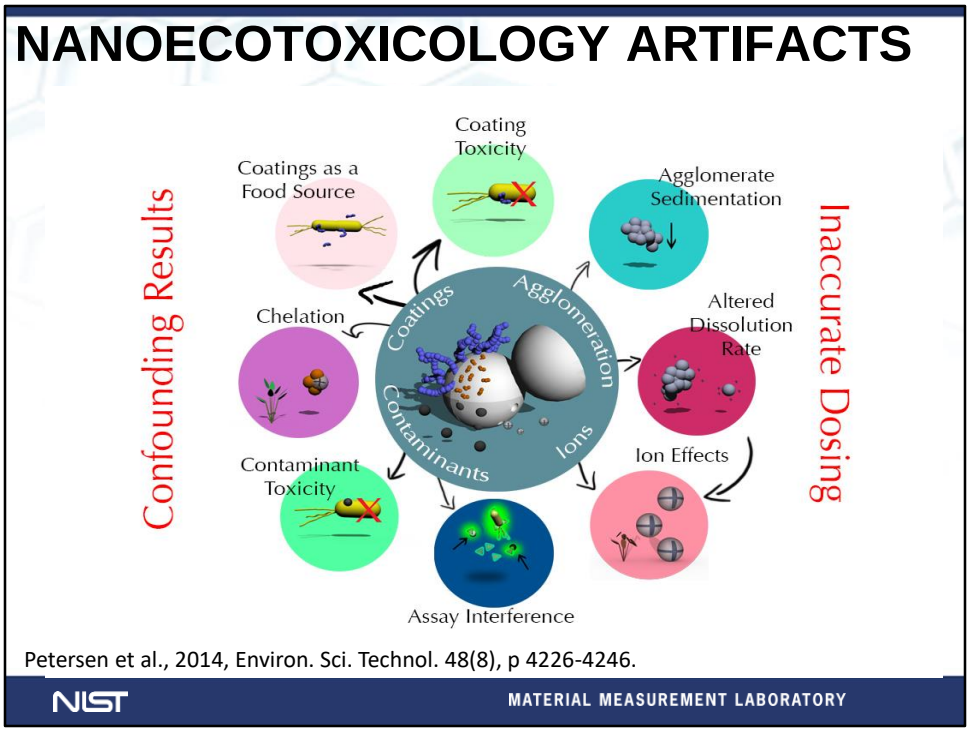
>> Elijah Petersen: I collaborated with a number of colleagues a few years back to attempt to systematically characterize these artifacts. In the coauthor list, there's a number of people from NIST, there's also people from different institutes, from the Federal family and state agencies, and also a lot of people from academic institutions.

So we went through and looked at, where could things go wrong? We found that they can happen on every step of the way. From the initial nanomaterials you procure, if there are changes during storage, dispersing the nanomaterials, or if they come from a powder to be suspended in aqueous media, there could be issues there. Measuring end points themselves, there could be interactions between the nanoparticles and test reagents, or the nanoparticles could have a signal similar to the absorbance and fluorescence read-out you're trying to measure with your test reagents, and there could be mischaracterizations of the NPs in tissues. In addition to characterizing the way things can go wrong, we also made a comprehensive list in this study of how can you test the things that might be going wrong, and what modifications you could make if there might be some of these problems. So there's solutions in addition to just categorizing some of the potential issues.



>> Elijah Petersen: This figure shows some of the potential different artifacts. For many nanomaterials, they are not stable in suspension by themselves, so often you have to add coatings to get the nanoparticles to be stable. But those coatings could be consumed by the organisms and be a food source, or they could potentially be toxic. There could be agglomeration or sedimentation of the nanoparticle, even if they may have a coating during the test, and that could lead to differences in the exposure during the course of test; where initially the organism may be exposed to one concentration, but later it could be exposed to a different concentration, or there maybe a substantial concentration on the bottom of the container. And depending on what species it is, some of them may be at the bottom of the container, some may be dispersed in the water column. It really varies a lot.

There could be dissolution of particles, especially for silver particles or copper oxide, and those ions that are released could potentially be toxic. Many nanomaterials also may have other compounds present. Potential contaminants, such as carbon nanotubes, may have metal catalysts present; those could be released and potentially be toxic. And as I already described, there could be interference with the assay itself from the nanoparticles.

>> Elijah Petersen: So with all this work, and the efforts that have been happening in the last 15 years, we, myself and the two collaborators: Al Kennedy from the U.S. Army ERDC (Engineer Research and Development Center); and Steve Diamond. Dr. Diamond was previously at EPA, then at Nanosafe, and now happily retired. So it's just Al and I at this point working on it. So with all this data coming in, we have gone through the consensus process and are trying to develop a guidance process for toxicity testing for the OECD test methods.

Why OECD test methods? Well, to my understanding in terms of environmental testing, often EPA and other agencies will use these methods, partly because there's something the OECD calls "mutual acceptance of data," or MAD, where if they use OECD methods in one country, the results will be accepted in other countries. Each country isn't requiring something different, which could really drive up the costs and limit the commercial opportunities.

Meetings

February 2014 – Vienna, Austria (University of Vienna)
July 2014 – Washington, DC (EPA, 23 experts from seven countries)
January 2015 – Dessau, Germany (German Environment Agency (UBA))
November 2016 – Paris France (Prosafe meeting)

Guidance document submission and revisions

First draft submitted to OECD September 2017
Revised drafts submitted to OECD August 2018, March 2019, and June 2019 (hopefully)

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>> Elijah Petersen: So for any of you who have been involved in documentary standards work, you know it involves both a lot of meetings and a lot of revisions. This started off back in 2014, in a meeting in Vienna, Austria. That was followed up with a second meeting in Washington, DC, at EPA headquarters, where we had 23 experts from seven countries. Then there was a meeting a bit later at Dessau, Germany, at their version of EPA, UBA. And finally a Prosafe meeting in November 2016.

We worked with all these experts to make our first version of this guidance document, and it was a really substantial effort, but we got it first submitted back in September 2017. It went back out, we got it back. Then we had some additional revised drafts in August 2018. Again, in March 2019, and hopefully later this month we'll get it back out for our third revisions.

Meetings

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>> Elijah Petersen: I think we're getting close now. I don't want to jinx ourselves, but we're getting fewer comments, and I think we are honing down on something that, while people may not agree with everything, it's gone far enough that we have been able to address the concerns such that people are getting increasingly comfortable with it.

But it's a process, and often different stakeholders may have different perspectives. So these efforts, they are extremely valuable, but they often take a while. And once this is developed, this will give the contract testing laboratories something to review and utilize. So, I want to do this fish test. Okay, so what's some guidance on how to test these substances that maybe more challenging than some dissolved substances in terms of applying the different test methods?

Environmental Science & Technology

Critical Review

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Adapting OECD Aquatic Toxicity Tests for Use with Manufactured Nanomaterials: Key Issues and Consensus Recommendations

Elijah J. Petersen,[†] Stephen A. Diamond,[‡] Alan J. Kennedy,[*,§] Greg G. Goss,[∥] Kay Ho,[⊥] Jamie Lead,[#] Shannon K. Hanna,[†] Nanna B. Hartmann,[▽] Kerstin Hund-Rinke,[○] Brian Mader,[◆] Nicolas Manier,[¶] Pascal Pandard,[¶] Edward R. Salinas,[△] and Phil Sayre[◇,+]

- Critically evaluated OECD aquatic toxicity test guidelines for use with nanomaterials
- There can be nanomaterial specific artifacts and the dimensions of the test container may have important effects on the assay results unlike for dissolved chemicals
- Highlighted numerous topics where consensus was and was not reached among the experts as well as recommendations to resolve issues where consensus was not reached

Petersen et al., 2015, Environ. Sci. Technol. 49(16), p 9532-9547.

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>> Elijah Petersen: As an intermediary output from this work we published a paper a few years ago where we critically evaluated the OECD aquatic test guidelines, looked at the different artifacts, and highlighted where there was some agreement, and also places where there wasn't agreement.

Now a lot of those have been resolved through additional discussion and additional research. That's supported us in getting to the place where we now have this guidance document ready.

Dosimetry emerged as a key issue

Many OECD test guidelines specified that the exposure concentration during the test or between renewals should change be less than ± 20 %.

However, it is unclear what concentration should be measured: mass, nanoparticle number, or surface area-based concentration?

Given the lack of standardized methods for measuring nanoparticle number concentration and surface area-based concentrations, the choice was made to prioritize mass concentration.

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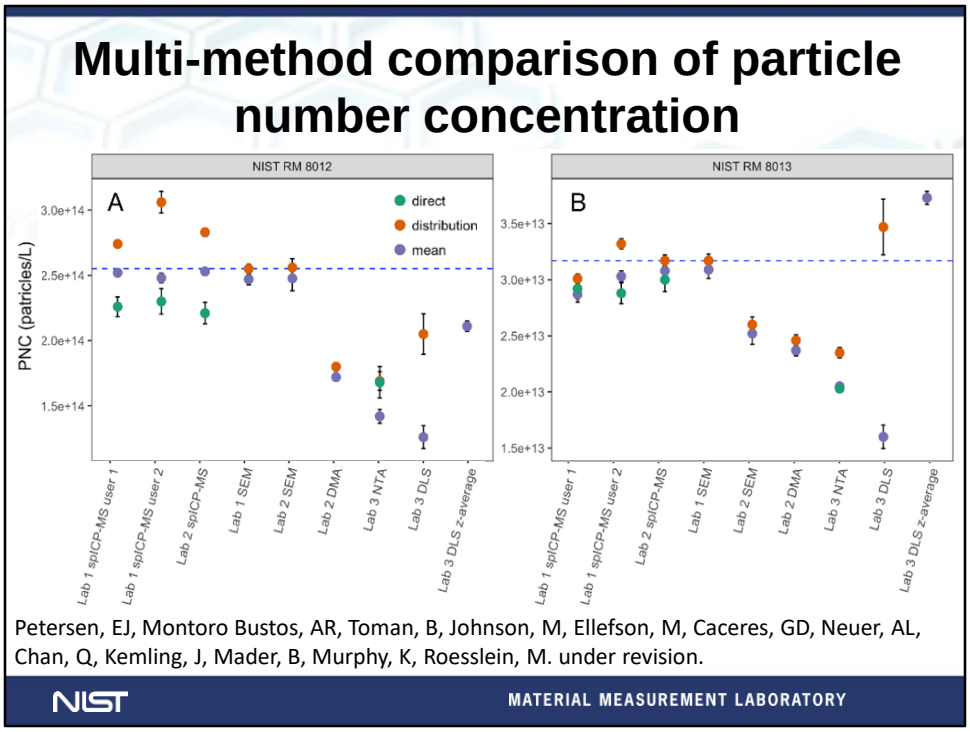
>> Elijah Petersen: One of the key issues that came up during the course of these discussions was dosimetry. How do you quantify what the organisms are being exposed to during the test? This is challenging because for many OECD test guidelines, they specify that the concentration can't change by more than 20%. But then for nanoparticles the question became, well, what concentration are you referring to? Is it the mass, the particle number, or surface area concentration?

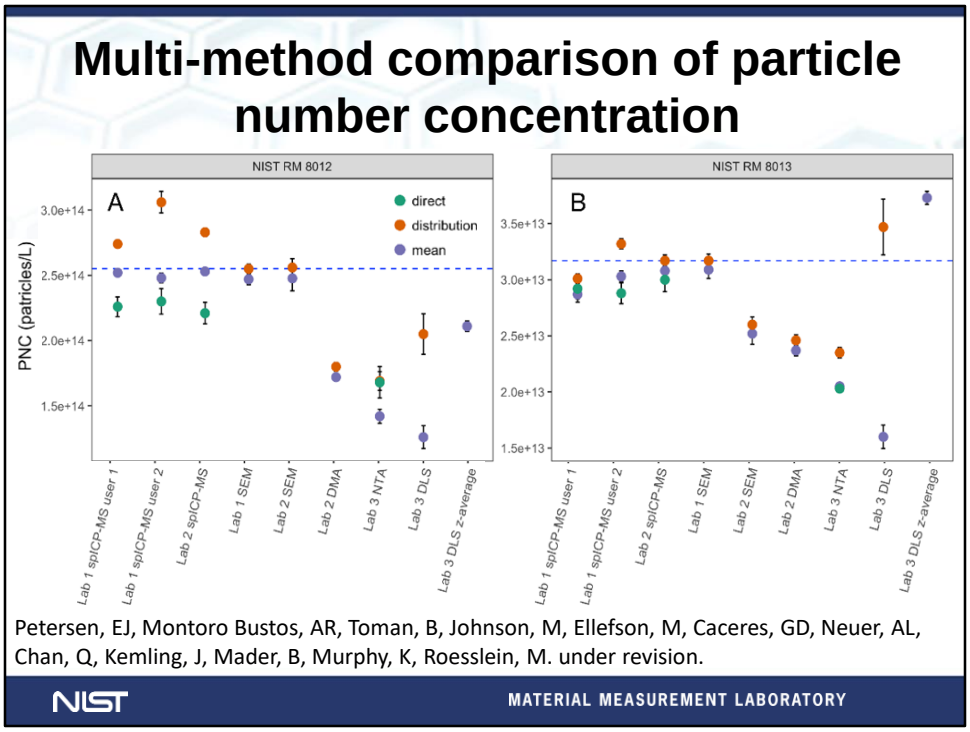
And based on which metric for the exposure, there could be different answers in terms of whether there was a 20% change or not. For example, if there was a nanomaterial that was staying in suspension and agglomerating, there could be a change in particle number concentration but not much of a change in the mass concentration.

Overall based on these discussions, what we came to was the choice to prioritize mass concentrations initially because there's good methods available for measuring this, and we think that for contract testing laboratories, we can already really get them good guidance, and there's been a lot of other test methods for physicochemical characterizations developed for this.



>> Elijah Petersen: Also, in a collaboration at NIST, we looked at some specific measurements of the nanoparticle number concentration. In particular we are curious about what is the variability among different laboratories for the same measurement over and among techniques? So I'll talk you through this. There's a lot of data here in this paper, I'm hoping to resubmit it this week, so hopefully it will come out soon. But it's been a really big effort between myself and a number of scientists at NIST, and scientists at different places. We have had collaborators who have been instrumental from 3M and also from EMPA in Switzerland.

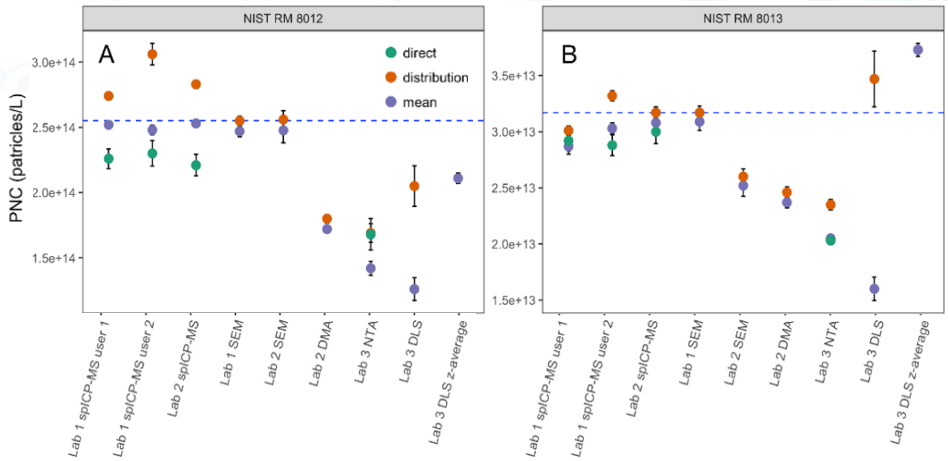
So let me talk about this graph a little bit to help explain it. Along the x-axis you will see there are different laboratories, different techniques, and for single-particle ICP-MS, two different users. The techniques we tested were spICP-MS or single-particle ICP-MS, scanning electron microscopy or SEM, differential mobility analysis or DMA, nanoparticle tracking analysis or NTA, and dynamic light scattering scattering or DLS. On the Y axis you see PNC, which is particle number concentration. In the study we tested four different gold nanoparticles. Well, why gold nanoparticles? Mainly because we thought they would be the easiest. This is a challenging measurement, so let's keep the type of particle simple.



>> Elijah Petersen: In the two plots I have here, this is the NIST RM 8012, which is a nominal 30-nanometer gold nanoparticle, and RM 8013, which is a nominal 60-nanometer gold nanoparticle, both coated with citrate. And we also tested in this study PVP or polyvinylpyrrolidone-coated nanoparticles or branched polyethylene (BPE)-coated nanoparticles. You will notice there are also three dots for some techniques here. There is a direct measurement because NTA and single-particle ICP-MS because they can directly provide a measurement of the particle number concentration.

And we also, for all these techniques, get a size distribution. With this size distribution and some other parameters, such as the mass concentration and density, it's then possible to derive a particle number concentration. Usually in the literature people use the mean value or some other central tendency indicator, typically the mean value, to calculate this. We found that there are some cases where using the full distribution made a substantial difference, especially if there was an asymmetric distribution for the size. You will also notice for the single-particle ICP-MS and SEM data, those seem to be in the closest agreement. That is where the blue dot goes across from the SEM measurement for the full size distribution. Often electron microscopy is treated as a gold-standard technique.

Multi-method comparison of particle number concentration

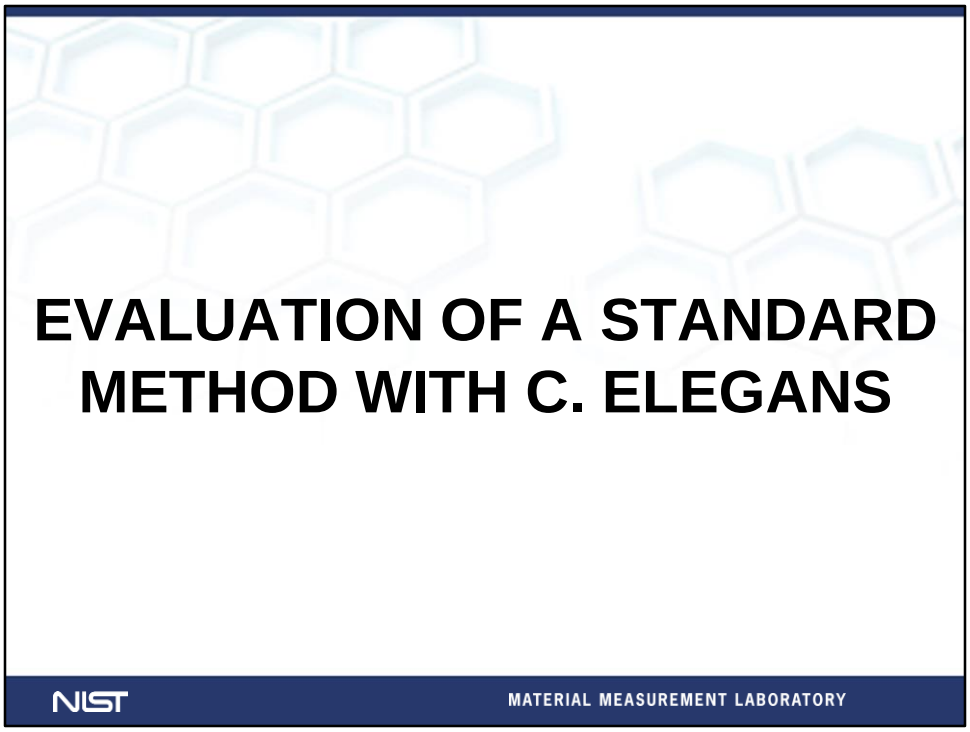


Petersen, EJ, Montoro Bustos, AR, Toman, B, Johnson, M, Ellefson, M, Caceres, GD, Neuer, AL, Chan, Q, Kemling, J, Mader, B, Murphy, K, Roesslein, M. under revision.

NIST MATERIAL MEASUREMENT LABORATORY

>> Elijah Petersen: The reason that SEM and single-particle ICP-MS results were in most agreement, we think, is because they measure just the core of the particle, not also the hydrodynamic diameter.

Overall what these data suggest is there could be substantial differences in the particle number concentration based on which technique you use, but also whether you use the mean or the full size distribution. Often this is treated as, well, probably wouldn't matter much, but some cases were found where it made a big difference whether you used the particle size distribution or just the mean particle size.



>> Elijah Petersen: In our work at NIST we also really focused on developing robust methods. I want to show a case study of that for a particular ecotoxicity method with *C. elegans*, a nematode.

Environmental Science Nano

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Cite this: *Environ. Sci.: Nano*, 2016, 3, 1080

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

S. K. Hanna,*[a] G. A. Cooksey,[a] S. Dong,[ab] B. C. Nelson,[a] L. Mao,[b] J. T. Elliott[a] and E. J. Petersen[a]

Shannon Hanna

Main focus was to evaluate the robustness of an ISO standard method with nanoparticles using a measurement science approach

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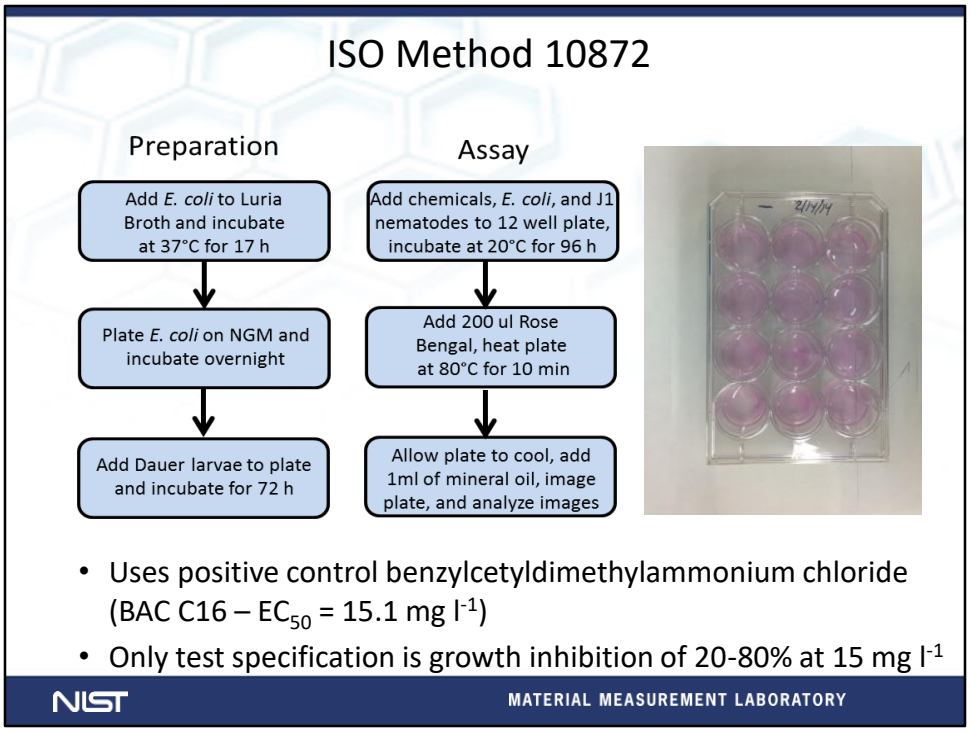
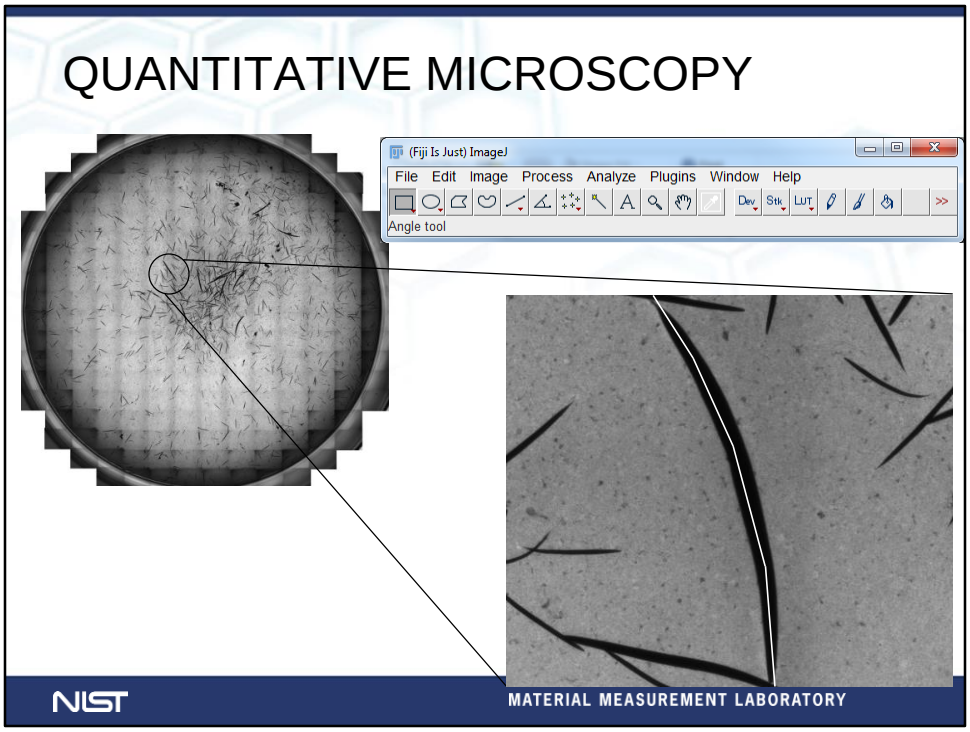
>> Elijah Petersen: This was developed as an ISO standard a number of years ago. We were basically looking at, well, how feasible is it to use this ISO method that was designed for dissolved substances for use with nanomaterials? The main person in the lab doing this work was Shannon Hannah, back when he was at NIST as a postdoc and then as staff scientist. He is now at FDA.

I'll talk about some of our findings and also our approach for really assessing whether the methods are robust, and provide some information about that.



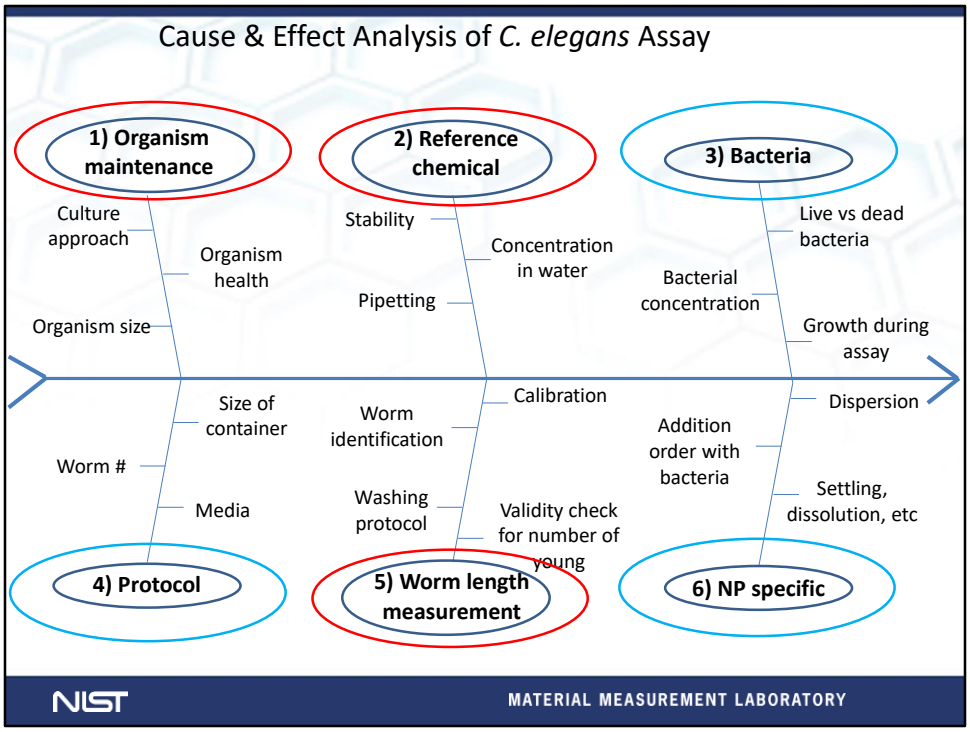
>> Elijah Petersen: One of the things we do when we get a method is we often will make a flowchart so we can specifically look at the different steps involved in the method. I guess it would be the “Assay” column, that describes specifically what we do in this assay.

Initially we will add the chemicals, *E. coli*, which is a food source for the *C elegans,* and J1 nematodes to a 12-well plate, then incubate at 20 degrees Celsius for 96 hours. Then, we'll add Rose Bengal to stain the worms, and then heat kill them at 80 degrees Celsius for ten minutes, which helps them straighten out. Normally they are often curved around, which makes determining their size more challenging. Then we allow the plate to cool and then add mineral oil and image them using a quantitative microscopy approach I'll describe shortly. In this assay there's also positive control, BAC C16, which is one of the only test specifications; you need to have the mode of inhibition of growth within this 20% to 80% range at the EC_{50} value for BAC C16.



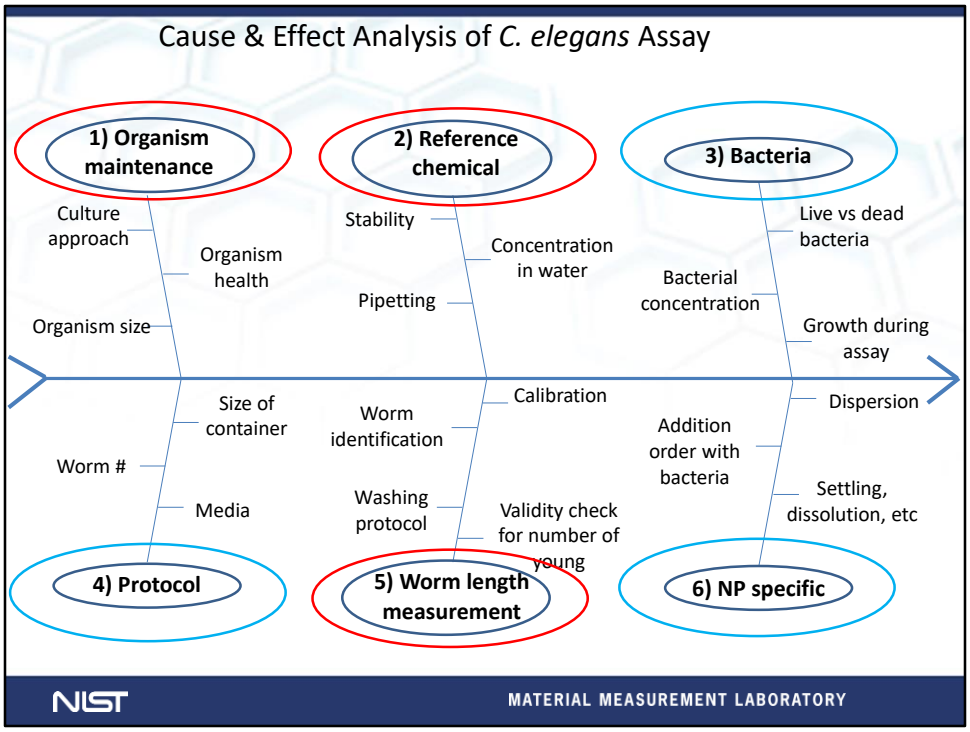
>> Elijah Petersen: Here is a slide just showing an example of quantitative microscopy approach that we developed at NIST. Every time we did this, instead of just looking through the ocular lens of the microscope and using the scale bar there, which you have calibrated, we wanted to take an image of the whole well every time we performed the assay, which then gave us archival data and allowed us to do some interesting image analysis. What we did is we took about 200 images, stitched them all together, then we had, for every well we tested, we had that saved.

Now, this is one of those slides where it looks simple but probably took two or three months of effort to get to this point. It was especially helpful to have the quantitative microscopy approach for worms like here where you see it is not exactly straight, but this would be hard to estimate a length if you couldn't make these segments and then add them together using *ImageJ* image processing software.



>> Elijah Petersen: Another common tool in measurement science that we apply at NIST is a cause and effect analysis. What's really nice about this is that it helps people go from having intuitive knowledge about a test or implicit knowledge. If you have been doing an assay for a while, you kind of know what things have an impact. This approach gets the information from being in someone's head to a piece of paper. It enables discussion among people about potential sources of variability.

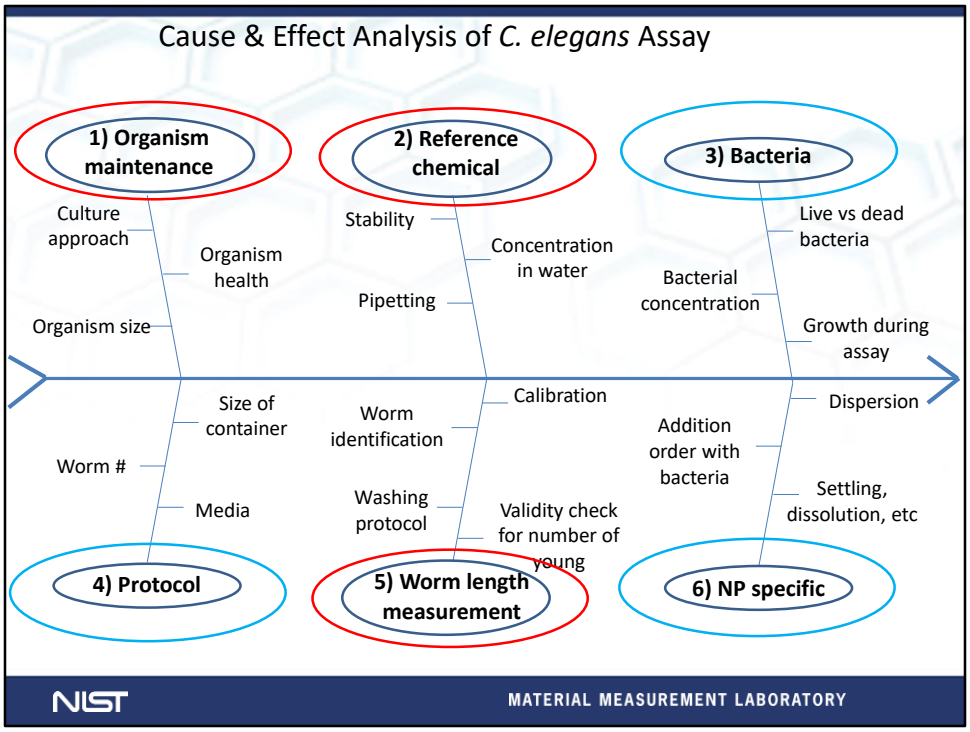
So what exactly are we looking at? I had six main branches or six main areas that could cause uncertainty in the assay. And within each of these branches we thought, “what are specific things within those branches that could cause variability in assay results?”



>> Elijah Petersen: So the first one is organism maintenance; it's possible to grow *C. elegans* in different ways. You could have grown them in liquid culture. You could have them on an agar plate. You want investigate whether that could that have impact. The reference chemical itself−−is there a difference among manufacturers? How stable is it in suspension?

Bacteria as a food source was key in this assay because a lot of nanoparticles are bactericidal. If you had, for example, silver nanoparticles and were toxic to the bacteria, and then the *C. elegans* are feeding on them, would that impact results? Does it matter if bacteria are alive or dead? How do you quantify the amount of bacteria you are adding?

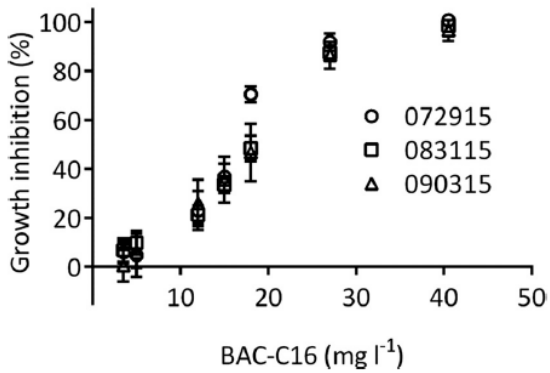
There's a few different approaches for the test media in the literature, and based on which approach you used, it could impact your result. In terms of the protocol, the ISO method suggests using the M9, which has a lot of chlorine present. Now if you were going to test the toxicity of silver nanoparticles, that could impact your results because the ions could be interacting with the chlorine to form silver chloride precipitate, or you could also be forming silver chloride particles.



>> Elijah Petersen: The worm length measurements, I already talked about that, and lastly, nanoparticle-specific issues. One thing we looked at in the assay is what happens if we shake the plate? Nanoparticles can settle during the course of the experiment, which could then change the exposure concentration. But if you could shake them, you could make the exposure more homogenous by keeping the particles in suspension.

So we did a whole bunch of robustness testing. We did eight experiments and repeated them twice. What we found is that some factors like these three (circled in red) didn't really have that much impact in terms of amount of variability, but the other factors (circled in blue) had a really big impact. Specifically bacteria concentration had a key influence. The media also was really important. And whether you shook the plate or not had an impact. So we suggested to not shake the plate.

Reproducibility with BAC-C16



EC_{50} for growth = 18.7 ± 2.6 mg l^{-1}

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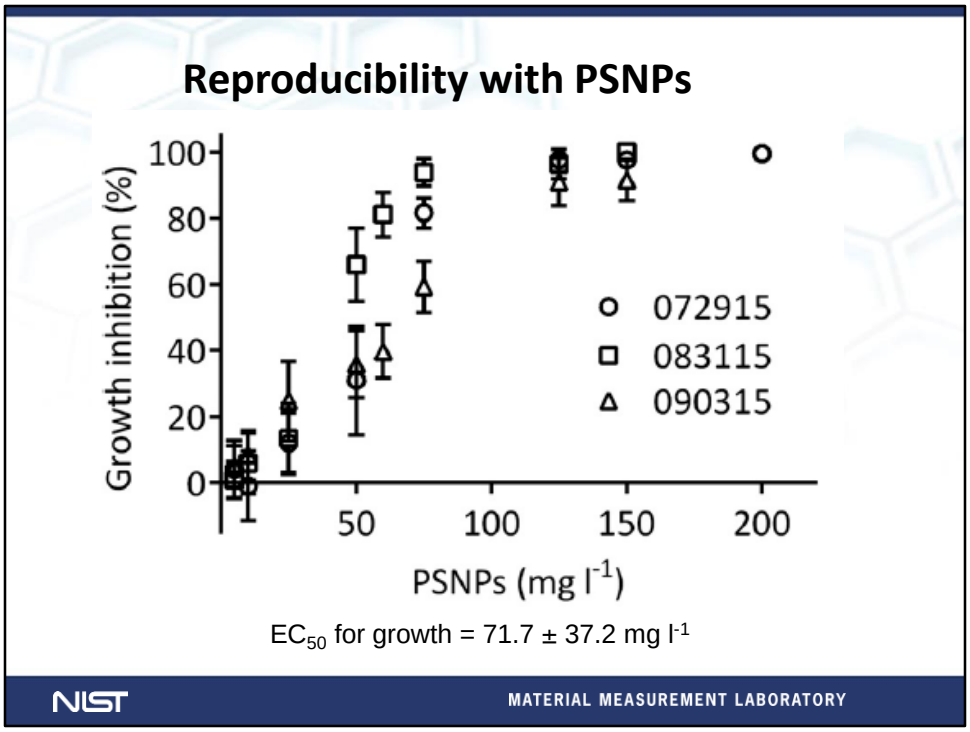
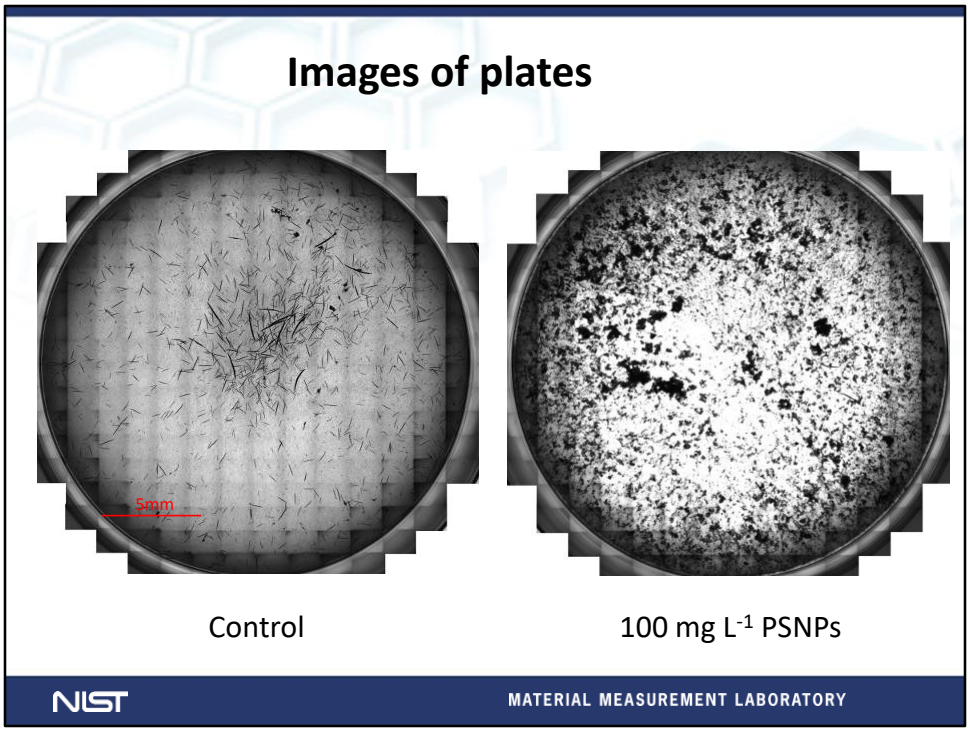
>> Elijah Petersen: For an example of what the data looks like from this assay, here is a figure of our reference chemical BAC C16. On the x-axis we have our BAC C16 concentration. On the y-axis here we have our growth inhibition. So basically, if there is zero growth inhibition that means organisms grew just as they normally would. If there was a hundred percent growth inhibition that means they didn't grow at all. They were the same size as the juveniles at the start of the experiment.

We were pretty happy with our EC_{50} value for this. Among the three different times we did the experiment was 18.7 plus or minus 2.6, which is about 15% coefficient of variation. That's looking good.



>> Elijah Petersen: But for the nanoparticles, our coefficient of variation was 50%, which had us scratching our heads a bit. And these experiments were conducted with polystyrene nanoparticles (PSNPs), which were positively charged with size of around 60 nanometers.



>> Elijah Petersen: But what was potentially causing the higher variability for the nanoparticles? One of the things we found was, looking at the plates afterwards, is well, the plates that had the polystyrene nanoparticles, they just looked different. Specifically, if you look at the control plate, you can see there's hardly any food or dark spots at the bottom of the well other than just the worms.

But for the polystyrene nanoparticles there are all these big spherical looking things. What we've hypothesized in this study is maybe the nanoparticles, which are positively charged, are interacting with the bacteria, which are negatively charged, and forming hetero-agglomerates. And then that's limiting the ability of the organisms to feed because the particles get bigger than their mouths are.

Environmental Science & Technology

Article

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Agglomeration of *Escherichia coli* with Positively Charged Nanoparticles Can Lead to Artifacts in a Standard *Caenorhabditis elegans* Toxicity Assay

Shannon K. Hanna,[*,#] Antonio R. Montoro Bustos, Alexander W. Peterson, Vytas Reipa, Leona D. Scanlan,[†] Sanem Hosbas Coskun, Tae Joon Cho, Monique E. Johnson, Vincent A. Hackley, Bryant C. Nelson, Michael R. Winchester, John T. Elliott, and Elijah J. Petersen

Is this assay robust when tested with a broader range of nanoparticles?

PSNPs – amine coated, 55nm

Si NPs – amine coated, 2nm

Au NPs – various coatings (PVP, PEG, Citrate, bPEI, dendron) and sizes (10-100nm)

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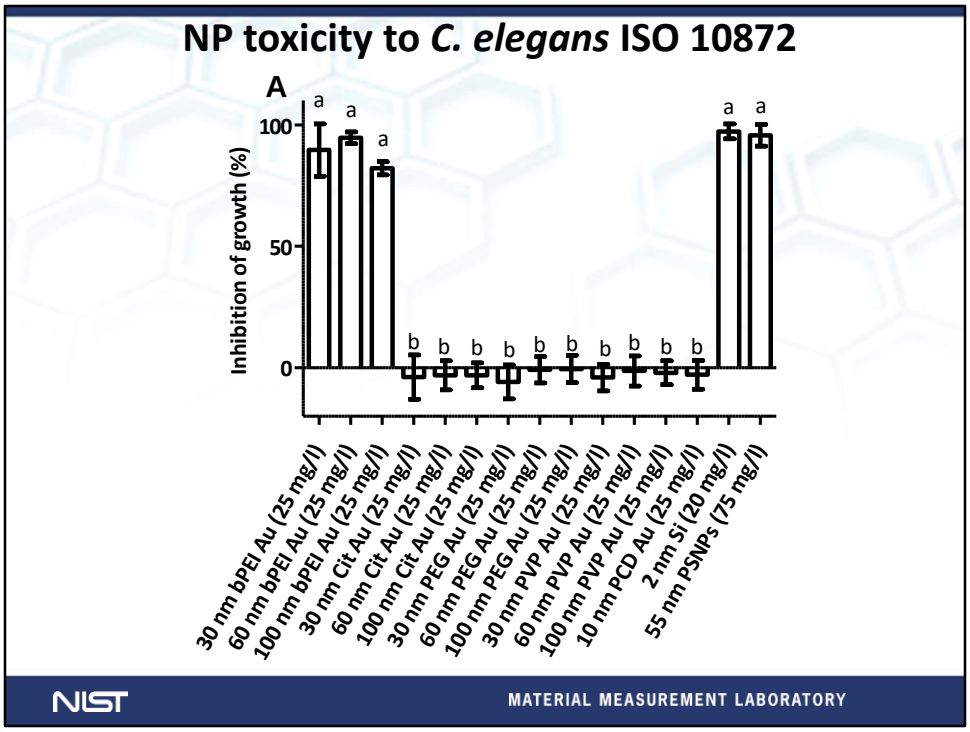
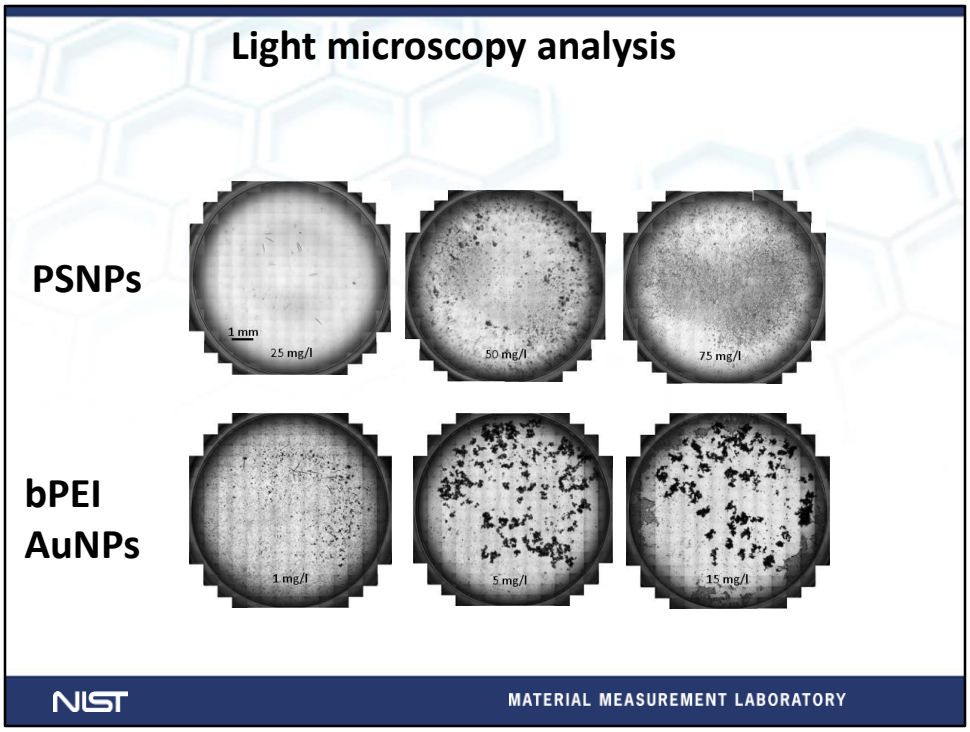
>> Elijah Petersen: Another dimension of looking at robustness of test methods is what's the applicability domain? Could it work with any type of nanoparticle? Are there some that would cause biases? In this study we then looked at about 15 different particles. We wanted to vary some different things; we varied the composition of the NPs. The ones we tested before were polystyrene nanoparticles. We also tested NIST reference silicon nanoparticles and a bunch of gold particles with different surface coatings. Specifically, the one I'll highlight is branched polyethyleneimine (bPEI), which is positively coated, as are the silicon nanoparticles in the range of sizes.

So what did we find when we looked at robustness of this assay for different types of nanoparticles?

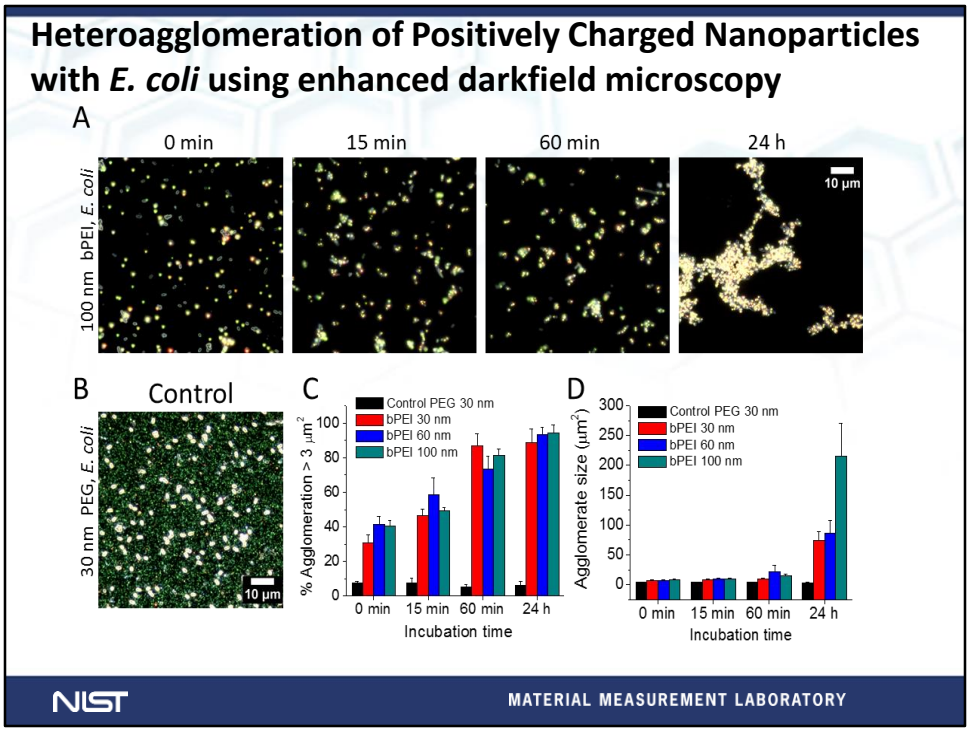


>> Elijah Petersen: What we observed was that our results were reproducible. We repeated everything at least twice. For many nanoparticles we noticed several that are near zero percent inhibition.

But there are five with near 100% inhibition. What we notice is all five that were at near 100% inhibition, they were all positively charged, similar to what we had the previous study with polystyrene nanoparticles.



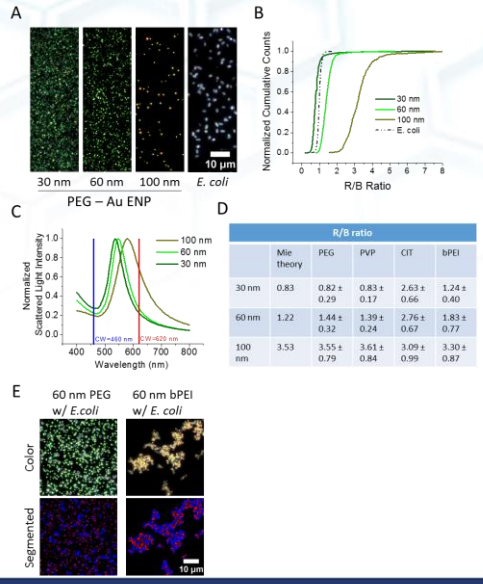
>> Elijah Petersen: What we found with these other nanoparticles, like the branched polyethyleneimine, is there were also hetero-agglomerates forming at the concentrations we were testing. So then we wanted to see what exactly is in these agglomerates? How can we characterize what's really happening there?



>> Elijah Petersen: So I worked with my one of NIST colleagues who is an excellent microscopist, Alex Petersen, and we used enhanced dark field microscopy to try to characterize what was happening.

So if you look at the top row you will notice that with these branched polyethyleneimine nanoparticles, with increasing time they start forming these agglomerates, and by 24 hours you have this fractal looking shape. In part B you will notice there is the control of polyethylene glycol nanoparticles, in which case there aren't any similar agglomerates. In Part C and D you can notice there was increase in the number of particles and in the size of those particles during the experiment up to 24 hours for the BPEI nanoparticles.

Heteroagglomeration of Positively Charged Nanoparticles with *E. coli* using enhanced darkfield microscopy



R/B ratio	Mie theory	PEG	PVP	CIT	bPEI
30 nm	0.83	0.82 ± 0.29	0.83 ± 0.17	2.63 ± 0.66	1.24 ± 0.40
60 nm	1.22	1.44 ± 0.32	1.39 ± 0.24	2.76 ± 0.67	1.83 ± 0.77
100 nm	3.53	3.55 ± 0.79	3.61 ± 0.84	3.09 ± 0.99	3.30 ± 0.87

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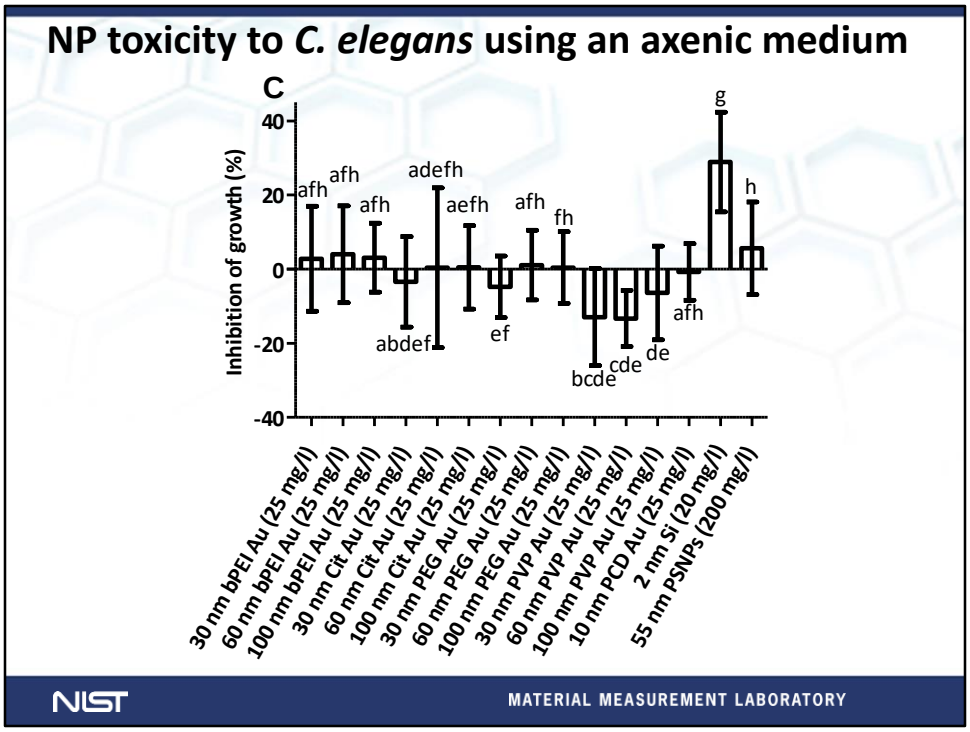
>> Elijah Petersen: So what exactly is in these agglomerates? To do that we looked at the different nanoparticles, and we wanted to look at, as you can see in part C the blue to red ratio, how much absorbance at different wavelengths. What we found, for the different size particles is that they varied in terms of the red to blue ratio, as did this ratio compared to that for *E. coli*. In part E, it's small to see, but look on the left, that's the pink particles. You will notice that the nanoparticles are in red and the bacteria are in blue.

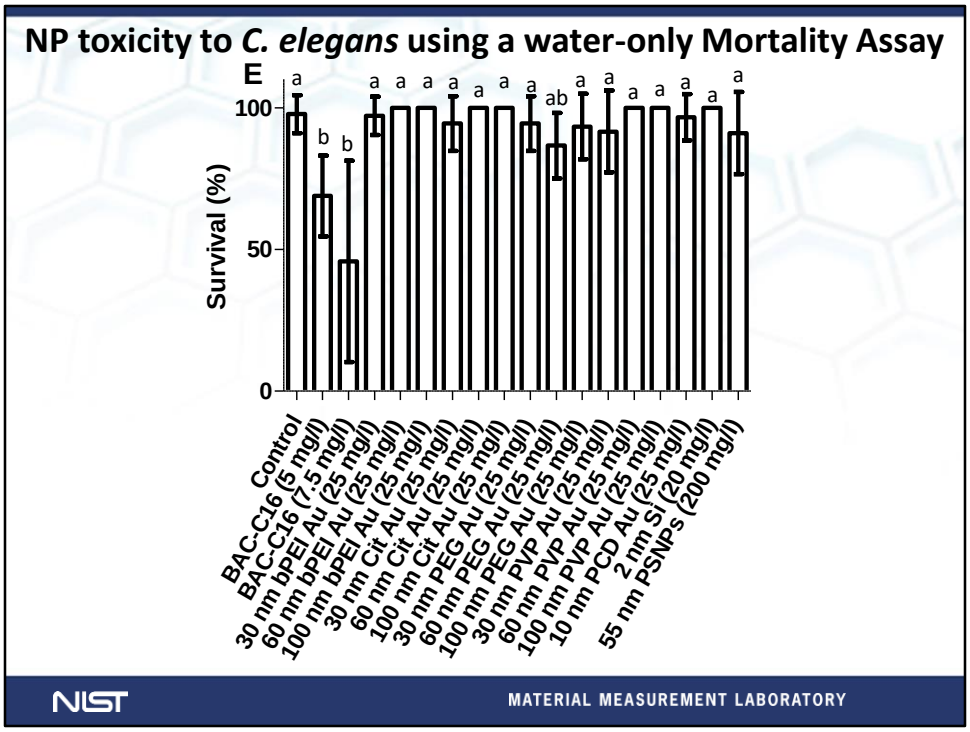
But there really is not any rhyme or reason, just a random distribution for the PEG particles. There's no pattern to be found. But in the branched polyethyleneimine ones, on right column in part E, you'll notice you're getting these fractal dimensions, and in those you see the red nanoparticles and blue bacteria, which really proved to us that there are these hetero-agglomerates forming.



>> Elijah Petersen: One of the things we also do at NIST is we look at orthogonal methods, or at least similar methods. So in this case we look at what if we didn't have bacteria present? What if we just have axenic media present? Now this is a complete food source for organisms, so if this is present, they wouldn't necessarily need the bacteria but they would grow bit more slowly but still do grow quite well.

Now you will see for most of them, there is no growth inhibition anymore, but for the silicon nanoparticles, there was still growth inhibition. And we are not really sure why that was the case. It could have been the small size. We're not sure.

But for the other ones, even if they were positively charged, it seemed like it wasn't impacting the results much. But one of the downsides of this axenic medium, or defined medium, is that there are many other substances present. There are proteins, cholesterol, and many another other things that are critical for the worms to grow.



>> Elijah Petersen: What happens if we just didn't have any of those other substances present? Just had a water-only exposure? In this case what we find is that there isn't any impact on the survival for any of the worms. But there is--we do see an impact from the BAC C16, which is our chemical control.

So overall what this suggested to us is that this assay--it really has some issues with positively charged particles because of this hetero-agglomeration, which could then lead to an indirect toxicity mechanism as a result of basically starvation and not having the food available for the worms. But for the other particles it seemed to work. We didn't notice any artifacts or biases.

COLLABORATION ON BIOACCUMULATION MEASUREMENTS WITHIN US-EU ECOTOXICOLOGY COMMUNITY OF RESEARCH

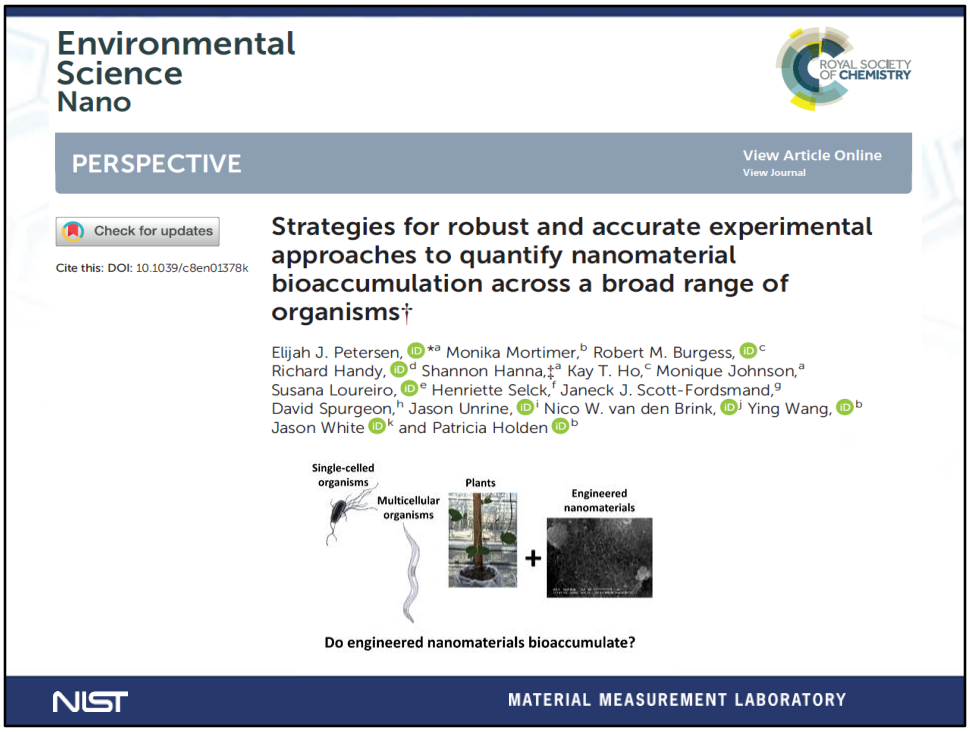
Elijah Petersen
NIST
(Former) US Co-chair

Nico van den Brink
Wageningen University
EU Co-chair

NIST MATERIAL MEASUREMENT LABORATORY

>> Elijah Petersen: And lastly I'll talk about work that's going on in the U.S.-EU Ecotoxicology Community of Research within the NanoEHS Bridging Research efforts groups. Hopefully most of you know of this, but it's a fantastic group where scientists from the U.S. and Europe get together. And we are able to share ideas, work on collaborative efforts. With so much going on in both places, how can we harmonize our efforts and have the biggest impact possible rather than both of us having similar things ongoing at the same time and not being aware of it?

I am the former U.S. co-chair and Adeyemi Adeleye is the current co-chair; he is at U.C. Irvine now as an assistant professor.



>> Elijah Petersen: I worked on this particular project on bioaccumulation measurements with Nico van den Brink as the EU co-chair. The next meeting for the group is October in France but I'm not 100% sure of the details. For this particular effort we wanted to look at, well, we have been doing research on this area for about 15 years. How can we come together and suggest some best practices and strategies for robust and accurate experimental approaches for nanomaterials across a broad range of organisms? Because there can be so many different potential organisms where there could be ecotoxicity bioaccumulation tests. You could have single-celled organisms like bacteria, multi-cellular organisms like nematodes that I just talked about, and plants. And each of those has different considerations.

This author list , there's a lot of people involved. And like many efforts I have shown, all of them were really essential for us get to this level because each of them had key knowledge and contributed in important ways. For this particular author list, it was about 50% in the U.S. and 50% Europe, so it really was a great collaborative research effort among our two groups.

Some key findings

Bioaccumulation terminology for NP studies is often inconsistent hindering comparison among studies, so terminology was suggested. There are also key differences compared to bioaccumulation of dissolved substances.

There are some substantially different methodological issues based on the type of organism tested: single-celled organisms, multicellular organisms, and plants. Case studies were provided.

Ongoing efforts to improve the analytical methods for quantifying NPs in tissues will enable a more detailed understanding of NP bioaccumulation by enabling detection at lower concentrations and comparisons among orthogonal methods.

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>> Elijah Petersen: So we had some key findings in this paper. I can't go into all of it now, but I'll leave you with a few highlights. One of the things we found that was that there is different bioaccumulation terminology for nanoparticle studies. So you need to be careful when comparing among studies, even in the nanoparticle area. Hopefully to alleviate this in the future, we suggested some terminology that hopefully the field adopts.

There's also some differences between nanoparticle bioaccumulation and that of dissolved substances, where for most dissolved substances like polycyclic aromatic hydrocarbons or metals, they can get across the gut tract relatively easily unless they are extremely hydrophobic or extremely large. That’s unlike nanoparticles, where a lot of them remain located in the gut tract. And then if you do bioaccumulation measurements and you don't have an elimination period, most of what you could be measuring could be nanoparticles packed in the gut tract; depending upon what's fit for purpose for your study, that may be really important.

Some key findings

Bioaccumulation terminology for NP studies is often inconsistent hindering comparison among studies, so terminology was suggested. There are also key differences compared to bioaccumulation of dissolved substances.

There are some substantially different methodological issues based on the type of organism tested: single-celled organisms, multicellular organisms, and plants. Case studies were provided.

Ongoing efforts to improve the analytical methods for quantifying NPs in tissues will enable a more detailed understanding of NP bioaccumulation by enabling detection at lower concentrations and comparisons among orthogonal methods.

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>> Elijah Petersen: As I was saying, there's also some really key methodological issues for different types of organisms. So we put together a number of case studies, where if you wanted to do experiments on single-celled protozoa, for example, or biofilms, here are some of the key considerations that you should really be thinking about. In the study we also focused on the analytical method portion of it, where as those keep improving, we can get to have increasing confidence in our measurements.

It's really challenging now because for some measurements we only have one good method. Even then we are pushing the method to its limit. In the future it would be really super to have orthogonal measurements because then we can potentially understand if one of the measurements is biased in some way that we otherwise may not be aware of. That's moving along; I think that’s a great opportunity for continued work.

Conclusions

Substantial progress has been made in improving the reliability of measurements of nanomaterial ecotoxicity. This has lead to the development of a GD for publication in OECD.

Many potential artifacts in nanoecotoxicology are now known and control experiments are defined.

There are convergent results in some areas of the field such as the lack of bioaccumulation of carbon nanotubes (Bjorkland, Tobias, and Petersen, Environ Sci. Nano, 2017, 747-766).

There are numerous additional efforts at standards organization (ASTM, ISO, and OECD) to support the accurate measurement of potential nanomaterial risks during the product life cycle.

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>> Elijah Petersen: To leave you, I have a few conclusions. The last one I'll say first. There's been so much progress. In this talk, I have been mostly focusing on efforts we have done at NIST on this topic, but there’s been so much work at different academic institutions, other agencies, in the U.S. and also internationally.

And while I focused on this again in this OECD document, there's really great work happening in ISO and ASTM that I didn't have a chance to cover today. And I think that overall, with all these different people involved, there's really super progress that has been made in the last 15 years. The point we have arrived at now, is where we're hopefully nearly ready to publish a guidance document for aquatic toxicity testing with nanomaterials through the OECD.

Conclusions

Substantial progress has been made in improving the reliability of measurements of nanomaterial ecotoxicity. This has lead to the development of a GD for publication in OECD.

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>> Elijah Petersen: There are, as I was saying, potential biases and artifacts. But now they are well known, and we have good control experiments to test for them to have information about that.

I didn't have a chance to get into this in today's limited time frame, but also there are some areas with really convergent results, where we have a good sense of what's happening. One of those is in a study that Rhema Bjorkland led back when she was an AAAS Fellow at EPA. David Tobias from EPA was also involved; where we looked at 40 to 50 studies of bioaccumulation of carbon nanotubes. And one of the things that was really valuable was there had been these orthogonal methods used. So we were able to say, could these findings have been the result of methods-specific bias? But because there were these orthogonal methods, we had more confidence in them.

Conclusions

Substantial progress has been made in improving the reliability of measurements of nanomaterial ecotoxicity. This has lead to the development of a GD for publication in OECD.

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There are numerous additional efforts at standards organization (ASTM, ISO, and OECD) to support the accurate measurement of potential nanomaterial risks during the product life cycle.

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>> Elijah Petersen: What we found for multi-cellular organisms: there was a consistent lack of bioaccumulation in these studies. Carbon nanotubes typically do not go through the gut tract at detectable concentrations, so it's nice when efforts lead to something we feel really confident about.

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Centre for Ecology and Hydrology
David Spurgeon

Nico van den Brink
Wageningen University

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>> Elijah Petersen: In this slide I have the huge number of collaborators who were involved, both at NIST and others; there are a number of people who were absolutely critical. And there's also these different studies; this is just for the studies shown here, but there's been other efforts too; it's really been a great collaborative effort.

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>> Elijah Petersen: These are references for six of the studies that I talked about. So far I don't have a reference up for the paper on the nanoparticle number concentration because it's not accepted yet. Based on that, we don't know the journal, but hopefully that will be coming out in the next month*. So if you are curious about that, when it comes out, I will be happy to let you know. Just send me an email. With that, thank you again for this opportunity; I would be happy to have any questions.

>> Treye Thomas: Elijah, thank you very much for that excellent presentation. Congratulations to you and your collaborators on the research, as well as this, hopefully, the guidance document. So that's a monumental effort. Congratulations again, on pulling that together. So for everyone we do have time for questions. One thing I do want to say before we move on, Elijah did mention U.S.-EU. That is available, the website is https://us-eu.org/. And we do encourage you to take a look at that. As Elijah mentioned, that has been a really fabulous collaborative effort between researchers within the U.S. and the European Union. So again, please submit your questions. I'll go ahead and read them to Elijah and he can feel free to respond.

* *14/8/2019 Petersen et al. 2019* doi: 10.1039/C9EN00462A

Q/A

Is NIST and/or the OECD EHS nanomaterials working group planning to develop guidance for measuring potential ecotoxicological risk in soils? Particularly from biosolids?

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>> Treye Thomas: We do have one, Elijah. “Is NIST and/or the OECD EHS nanomaterials Working Group planning to develop guidance for measuring potential ecotoxicological risk in soils? Particularly from biosolids? “

>> Elijah Petersen: Thank you, that's a great question. I think one thing I didn’t mention in this OECD toxicity guidance document: we do have a few sections related to sediment ecotox testing. I think that a lot of the same key points relevant for the sediment ecotox testing will also be key for soils. We had discussed whether to include soils as well. Often, for a lot of products they typically look at three main species, three different tropic levels: algae, *Daphnia*, and fish. That's what we mainly had to focus on. We don't have any specific efforts on this, but I believe that the input from the sediment discussion would readily carry over though, even though testing in soils wasn't a specific focus.

Q/A

Has the influence of size of nanoparticles been investigated, particularly for charged or anti-bacterial particles?

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>> Treye Thomas: Okay. Great. We do have another question. “A very interesting talk. Has the influence of size of nanoparticles been investigated, particularly for charged or anti-bacterial particles?”

>> Elijah Petersen: Thank you for that question. I can, with some confidence, talk about the *C. elegans* method that I just discussed. And in that study, surprisingly, we didn't see any. Well maybe not surprising, but we didn't see any size-related effect, even though we tested particles from one to one hundred nanometers. For other studies, my impression is that in the literature, it would vary. There are a lot of important dimensions, such as that the size, and specifically the surface area, of the particles can influence the dissolution rate, which could influence the anti-bacterial effects on, for example, silver nanoparticles. But I wouldn't be comfortable hazarding a general statement of the impact of size. But I will say there's a number of studies that looked at it, and in ours, we didn't find effects, but in many others they have.

Q/A

In the bioaccumulation of carbon nanotubes, what was exposure the method?

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>> Treye Thomas: Great. More questions are coming in. This one is fairly short. “In the bioaccumulation of carbon nanotubes, what was the exposure method?”

>> Elijah Petersen: Thank you. I neglected to mention that. It really varies based on the study. There were a number of studies where the carbon nanotube source was added to food, and then the organism was consuming the food. There were also a number of studies where the nanomaterial was added directly to soil or sediment or suspended in water, and then bioaccumulation was measured. I don't think there were dermal absorption studies that I can recall, but at least for ecotox, most of the other exposure methods were investigated. There’s been some work on inhalation and biodistribution after inhalation, but I don't know those results off the top of my head.

I will say that there was also a pretty broad range of organisms that were tested, from protozoa, to fish, and earthworms--a whole bunch of species and many in-between. So we had more confidence in results since such a broad range was tested.

Q/A

How does hetero-agglomeration of bacteria induced by positively charged nanoparticles compare with biofilm formation?

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>> Treye Thomas: Great. Another question. "How does hetero-agglomeration of bacteria induced by positively charged nanoparticles compare with biofilm formation?"

>> Elijah Petersen: A good question. So in this study, I think the situation would be different if there weren't the *C. elegans* present. So the *C. elegans*, they are crawling around on the bottom of the wells, and when bacteria get down there, they tend to just eat them up pretty quickly. There weren't any really visibly detectable clumps of bacteria present for our control wells. Now, this would have me think that there wouldn't be enough time for a biofilm to be formed because the *C. elegans* would be consuming those bacteria. In terms of other scenarios, where say the *C. elegans* weren't present, could the hetero-agglomeration influence biofilm formation or support it? In this case, I don't really know enough to say how that would influence biofilm formation, but I don't think we were observing it in our study of *C. elegans*.

Q/A

Treated wood products now use copper nanoparticles. These could work their way into soil and could be toxic to organisms. Is this being looked into?

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>> Treye Thomas: Okay, another question. I may have to help you answer this one.

>> Elijah Petersen: Yes, please!

>> Treye Thomas: "Treated wood products now use copper nanoparticles. These could work their way into soil and could be toxic to organisms. Is this being looked into? "

>> Elijah Petersen: The reason Treye said that is that he's been involved in a large, multiple government agency collaboration on this very topic. My recollection from some of the aquatic sediment studies that were done at EPA and other agencies is that the toxicity was similar to the amount of dissolved copper that was released. But that's just my recollection, I don't have that study in front of me. Treye, would you please add more? Is my recollection correct?

>> Treye Thomas: I can just give a little background. As you mentioned, this is a collaborative effort between the U.S. Consumer Product Safety Commission, the Environmental Protection Agency. NIOSH was involved, as well as NIST.

Q/A

Treated wood products now use copper nanoparticles. These could work their way into soil and could be toxic to organisms. Is this being looked into? (Continued)

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>> Treye Thomas: Initially we were looking at the hand wipe method to determine if there's migration of the copper nanoparticles out of the wood during human contact. For example, you are rubbing or sitting on the deck. There are also subsequent studies, as Elijah mentioned, looking at leaching out of the material and into the soil. Those reports are available. They have been published in the peer-reviewed literature. There's also a report, I believe available on the EPA.gov website. It was published as an EPA report. And the lead author was Dr. Todd Luxton from the EPA.

We continue to look at surface wood coatings; cerium oxide as well. At NIOSH they look at the release of the wood during cutting--dust and so forth. So again, it's a fairly comprehensive study, looking at materials that are in the wood, pressure treated, as well as surface coatings to protect the wood. So again, I encourage you to look for those articles. You can contact me if you cannot find them.

>> Elijah Petersen: Thank you Treye.

Q/A

In your study, did you evaluate toxicity using zebrafish?

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>> Treye Thomas: Okay. Another question. “In your study, did you evaluate toxicity using zebrafish?”

>> Elijah Petersen: Well, thank you. I don't know, for the OECD guidance document. I don't recall offhand what species were included for potential testing. I would need to look up the OECD acute or chronic toxicity studies with fish to see if zebrafish would be included there.

There was some discussion in the guidance document for specific methods related to fish, and I believe that those findings would also be relevant for zebrafish studies, in addition to if there were testing with zebrafish embryos and the effect tests. But I can't say more because I don't have that directly up in front of me to say with more confidence than that. The overall findings and suggestions should support measurements of toxicity using zebrafish.

Q/A

Is it possible now to use the information on sources of artifacts and bias in a computational way to iron out conflicting results?

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>> Treye Thomas: >> Great. “Is it possible now to use the information on sources of artifacts and bias in a computational way to iron out conflicting results?”

>> Elijah Petersen: Thank you; a very good question. I think the answer is “it depends,” but I will give more details on that. For some artifacts, such as, for example, in some of the earliest studies looking at the toxicity of fullerenes, suspending them using tetrahydrofuran, it has been well documented now that there were some byproducts that were formed from that dispersion process that could influence the toxicity results. In that case, if there's conflicting results from C60 fullerenes suspended in water versus THF (tetrahydrofuran), we have enough information to sort that out. In terms of other areas, it's case-specific. I do think that in the literature the quality of the research and incorporation of control measurements has substantially increased. Information coming out now compared to things published back in 2005–really is light years better. I think there is still some ongoing refinement/improvement, but it's really come a long way.

Q/A

Is it possible now to use the information on sources of artifacts and bias in a computational way to iron out conflicting results?

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>> Elijah Petersen: One of the challenges, in terms of the computational area, is if people haven't used one of the standard methods, for example, EPA methods. It so easy to make a few different choices, like well, maybe I'll go for a little longer, I'll do a few more organisms, I'll change this or that. Typically there's not a lot of robustness testing with these methods. So say, for example, I went two days instead of four days--how can we combine these results?

It gets really challenging because there's variability just from different laboratories. There's variability from repeating within a laboratory, and there is variability from using different methods. In the absence of robustness testing, it's really hard to sort out some of those. But if they have used the test methods and adhered to it pretty strongly, I think in that case you are pretty far along, and there's a good chance for comparability then.

Q/A

Does NIST have a method for determining number of copper nanoparticles per gram of soil?

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>> Treye Thomas: “Does NIST have a method for determining number of copper nanoparticles per gram of soil? “

>> Elijah Petersen: I would say not exactly. I wouldn’t say NIST has a method. I know some work of my colleague, Vince Hackley, who was looking at methods to extract gold nanoparticles from soils using, I believe, a cloud-point extraction method, that was published in the last year or two. I don't recall if this was also applied to other nanoparticles or not, so I can't talk about that. And with that being said, a few general thoughts about this measurement challenge is that you are going to have uncertainty from your extraction procedure, and then you'll have uncertainty from, are the copper nanoparticles transforming in the soil? Separating out some of the different sources of uncertainty can be really tricky.

Q/A

Does NIST have a method for determining number of copper nanoparticles per gram of soil? (Continued)

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>> Elijah Petersen: So I think you could have a good measurement of copper, the total copper in your soil. You can have some good measurement of the amount of dissolved copper and transformations, but you may need to do x-ray absorption spectroscopy that may not be available except for in some specific laboratories.

But to do the extraction and to sort out, if my recovery is low, or why is that the case? Is the recovery low because there's agglomerates? Then to go from that to a number for concentration, I say it's definitely a helpful thing to think about, but it wouldn't be an easy measurement.

Q/A

Have you done any transcriptomic studies to see how nanoparticles affect gene expression?

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>> Treye Thomas: We have couple more questions. “Have you done any transcriptomic studies to see how nanoparticles affect gene expression? “

>> Elijah Petersen: Interesting. I think that looking at some of these more subtle end points and omics-based end points, there's a lot of promise to that in the future, especially when you start to look at adverse outcome pathways, trying to understand the mechanism of the toxicity instead of just, well, so many *Daphnia* died, and so forth. I think that at NIST we have done some work on different types of omics and transcriptomics measurements, and I think we have a reference materials that we have designed that can help increase confidence in transcriptomic results.

Q/A

Have you done any transcriptomic studies to see how nanoparticles affect gene expression?

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>> Elijah Petersen: So if you are personally interested in those measurements, I suggest looking into that. I would be happy to help connect you with some references, and what references were discussed in inter-laboratory comparisons, and what reference materials may support that.

In terms of myself, I have not been involved in any transcriptomic studies with nanoparticles and gene expression. I know in the literature there's been a lot of studies on this topic. And I can't summarize them offhand, maybe even if I look them up I couldn't summarize them, but there's been valuable work done on this topic by others.

Q/A

Are there examples of how this work is impacting safe design/green design, of nanotechnology enabled products?

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>> Treye Thomas: We have a couple more questions. “Are there examples of how this work is impacting safe design/green design, of nanotechnology-enabled products?”

>> Elijah Petersen: I'm not personally involved in the safety design, green design side of things. I think that by having robust, trustworthy methods, it's going to help people make choices earlier about, oh, maybe the carbon nanotubes should have this surface coating or not that surface coating. Or maybe for some applications, nanoparticles maybe safer or less likely to be released potentially leading to exposure. Unfortunately since I'm not specifically on the safety design and green design side of things, I don't have specific examples other than to say you need to have good methods that are robust to support these decisions. Otherwise there could be potential biases, which would influence you and steer you in a direction that is not the safest particle.

Q/A

Are there examples of how this work is impacting safe design/green design, of nanotechnology enabled products? (Continued)

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>> Elijah Petersen: Treye, do you know of any specific examples of this by chance?

>> Treye Thomas: You know, again, I think in general, I would say having robust studies and having the information--I'm certainly aware of users of the information, and in fact in 2013 we had a conference, the R3 conference (see https://www.nano.gov/r3report) where we actually have some presentations by organizations that were using some of the data and determining whether materials were safe for use.

So those efforts are ongoing, and I think again, as you pointed out, the important factor is the availability of the data. I think that it is great that we are moving forward and--there are sufficient data to conduct those types of analyses.

Q/A

What would you consider as the current top challenges for ecotoxicity testing?

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>> Treye Thomas: We have a minute left for one last question. “What would you consider as the current top challenge or challenges for ecotoxicity testing?”

>> Elijah Petersen: I think two things come up off the top of my head. One of them would be the dosimetry portion. For some studies, especially on nanoparticles that maybe more toxic, it could be challenging to get to have good quantitative methods for the full range of exposure conditions that you want to test. And then you run into issues with, well, then do I extrapolate from the concentrations above my detection limit to go to lower ones? That does complicate things.

I think another challenge from the NIST perspective is the inter-laboratory comparability. In this guidance document we are giving general suggestions and we are providing the best input we have, but this effort didn't specifically include inter-laboratory testing and transferability. I think that would be another really valuable thing in terms of the regulatory testing dimensions.

Concluding Remarks

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>> Treye Thomas: Okay. Well, looks like unfortunately we are out of time. It's been really a terrific webinar. We appreciate, Dr. Petersen, your knowledge and sharing your knowledge with us. Again, we congratulate you on the guidance document. So, thank you. To all those who participated, we appreciate you taking the time out of your schedules to participate in this webinar.

Again, this will be posted. The slides and information will be posted on the nano.gov website. I encourage you to go to website and to look at the general information available, and also, as we mentioned, the 2011 NNI Environmental, Health, and Safety Research Strategy. With that, we'll end. Everyone please have a great afternoon. Thank you and look out for our next webinar.