Enhancing Protocols to Improve Consistency Across Nanotoxicology Studies

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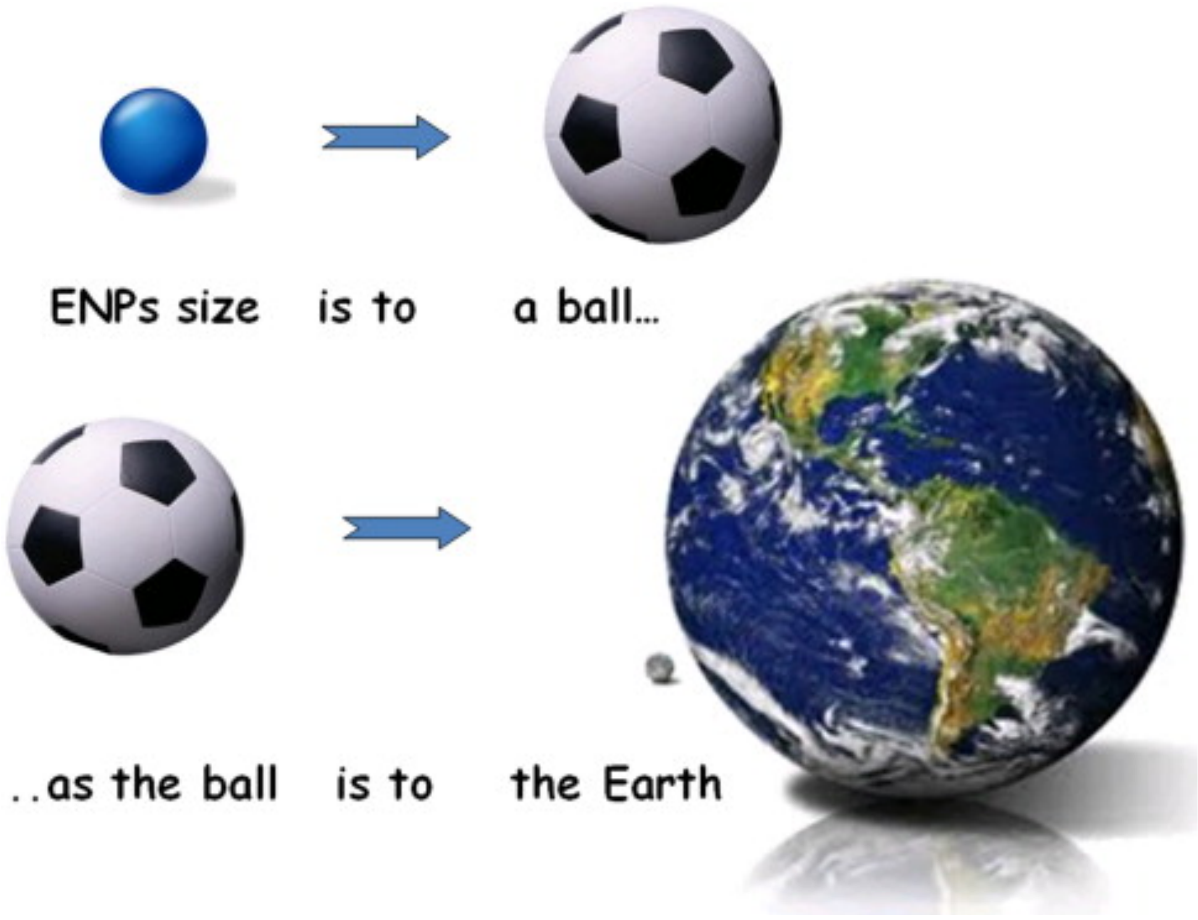
Nanotechnology

- Continuously growing field
- Application in biomedical, pharmaceutical and commercial industry
- Many Nanoparticles (NPs) are already in use and have not been evaluated for toxicity
- Important to study in toxicology as this could lead to potential human health and environmental risk

Current Commercial Uses:

Nanoparticles (NPs)

- 1 – 100 nm
- Have different shapes, sizes and chemistry
- Can have the same chemical composition as their larger counterpart
- Differences in NPs can influence their potential toxicity



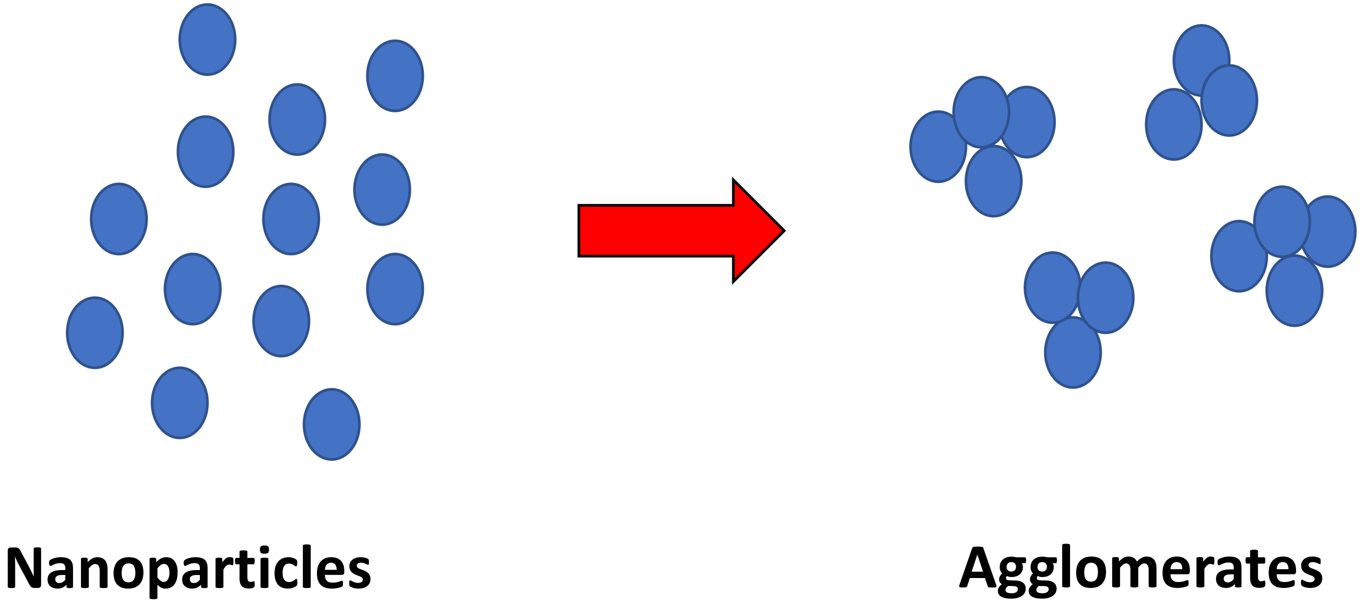
Toxicity Testing- Zebrafish Model

Advantages of Zebrafish Model

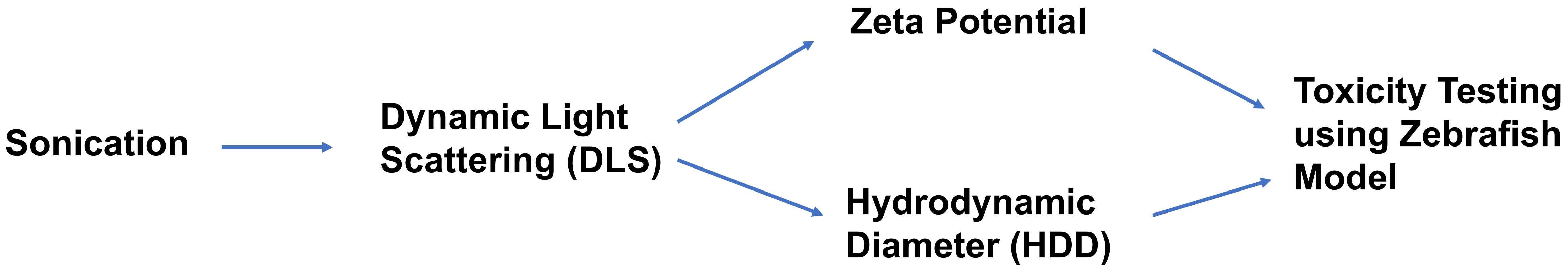
- Cheap
- Fast
- Streamlined
- Transparent bodies
  - Allows for easy observation of morphological and histopathological changes
- Conserved genomes
- Quick development

Issues with Toxicity testing of Nanoparticles

- Agglomeration Impacts:
  - Size
  - Surface area
  - Behavior
  - Toxicity
- Agglomeration causes:
  - Increase in size
  - Decrease in surface area
- Agglomeration is impacted by the way NPs are dispersed (sonication)



Characterization of Nanoparticles



- **Dynamic Light Scattering (DLS)** works by shining a laser through a suspension that determines a particle's size by measuring random changes of light within the suspension

- **Zeta Potential** relates the surface potential to the surface charge and impacts agglomeration which gives a way to look at the stability of the sample

- **Hydrodynamic Diameter is** the size and size distribution

Zebrafish Exposure Media Study- Introduction

- Current lack of standardization in reporting and use of Zebrafish exposure media in nanotoxicology studies

- Literature review showed that many studies did not report media used or reported minimal information to allow the recipe to be replicated.

- Media that interacts favorably with Zebrafish and nanoparticles is important when conducting studies

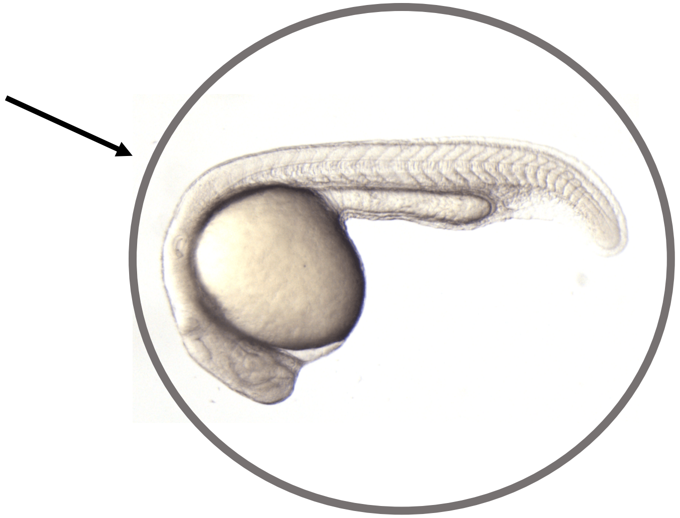
- Exposure media used can impact toxicity results and cause difficulty with reproducing results between labs

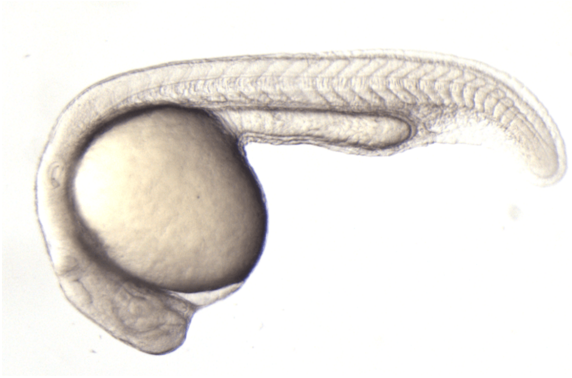
Fish Water Media Components

Different components of an exposure media can impact the behavior of a nanoparticle

Recipe	$CaCl_2$ (mM)	KCl (mM)	KH_2PO_4 (mM)	$MgSO_4$ (mM)	Na_2HPO_4 (mM)	NaCl (mM)	$NaHCO_3$ (mM)	Buffer
Media 1	0.33	0.17		0.33		5.0		
Media 2	0.76	0.67				59.0	2.4	
Media 4		0.23		0.13		19.3		1.67 HEPES
Media 7	1.3	5.4	0.44	1.0	0.25		4.2	
Media 10	1.0	0.5	0.15	1.0	0.05	15.0	0.01	

Fish Water Media Exposure





Chorion On

- NPs can settle on and clog the chorion pores
- Causing Hypoxia and death during nanoparticle exposure

Chorion Off

- NPs settle onto the embryos at the bottom of the 96 well plate
- Recommended method for Nanotoxicology studies

Fish Water Media Study- Objective

- To characterize and implement a fish water media recipe to replace the fish water currently used in our laboratory (Instant Ocean)

Methods:

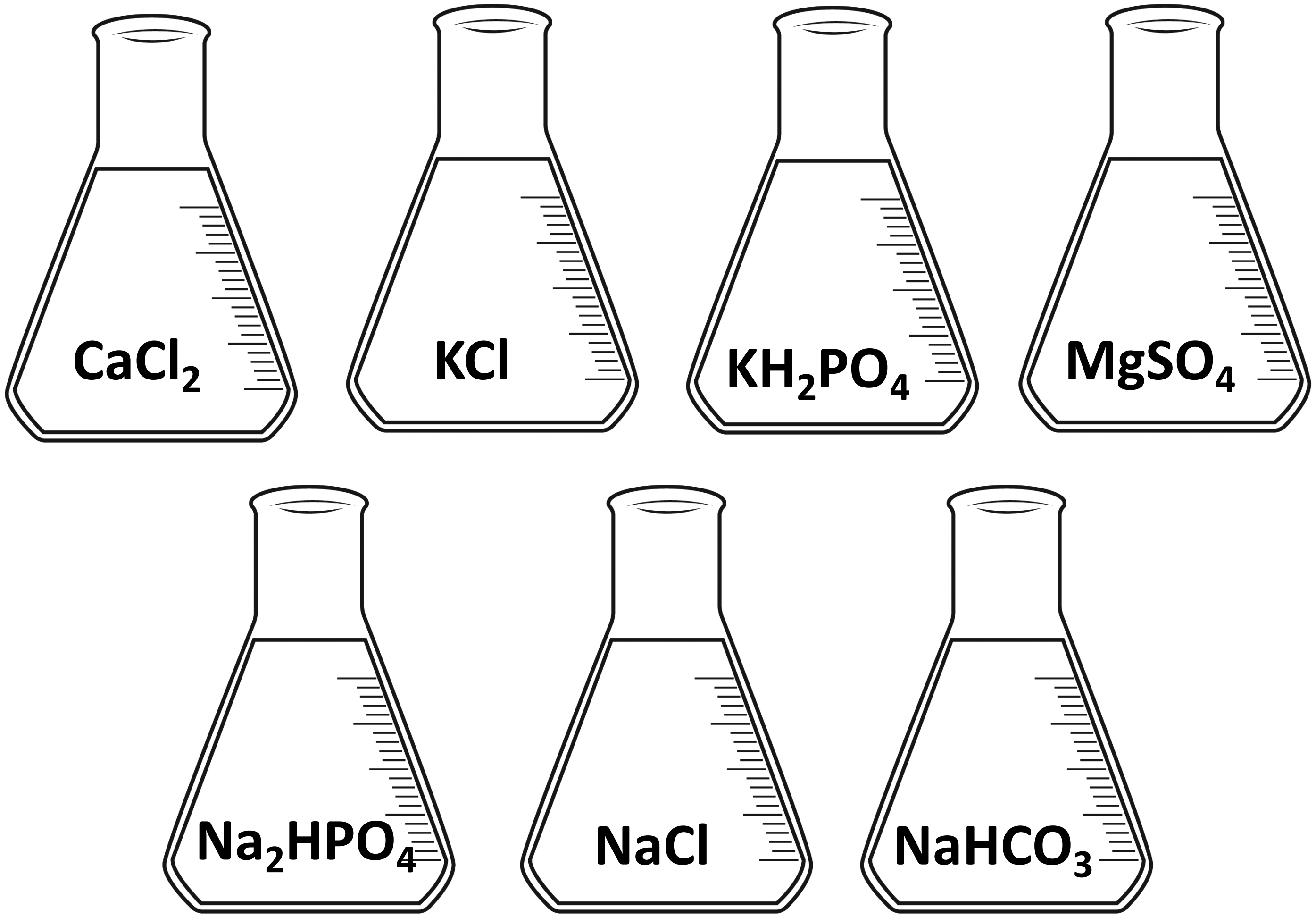
Literature Review → **Fish Water Media Stock Preparation** → **Sonication and Dynamic Light Scattering (DLS)**

Literature Review- Methods

- Focused on current published nanotoxicology studies to look for reporting of recipes for Zebrafish exposure media
- Found 11 medias reported, cited, or used
- Selected 5 of the media recipes based off thoroughness and ability to replicate the recipe for study

Fish Water Media Stock Preparation- Methods

- 1.67 mM HEPES buffer was added directly to the media
- Required amount of each stock was combined with ultrapure water to form a completed media
- Each media recipe was vortexed, then tested for pH and conductivity
- Stock solution of TiO_2 NPs was created

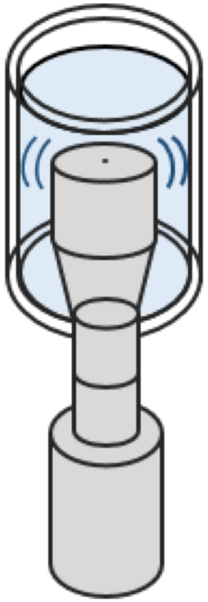


Sonication & Dynamic Light Scattering- Methods

Ultrasonication:

- TiO_2 NPs stock was sonicated for 2 min. at 40% amplitude

- NPs were placed into the 7 different medias and sonicated at 2, 10 and 30 min. at 40%

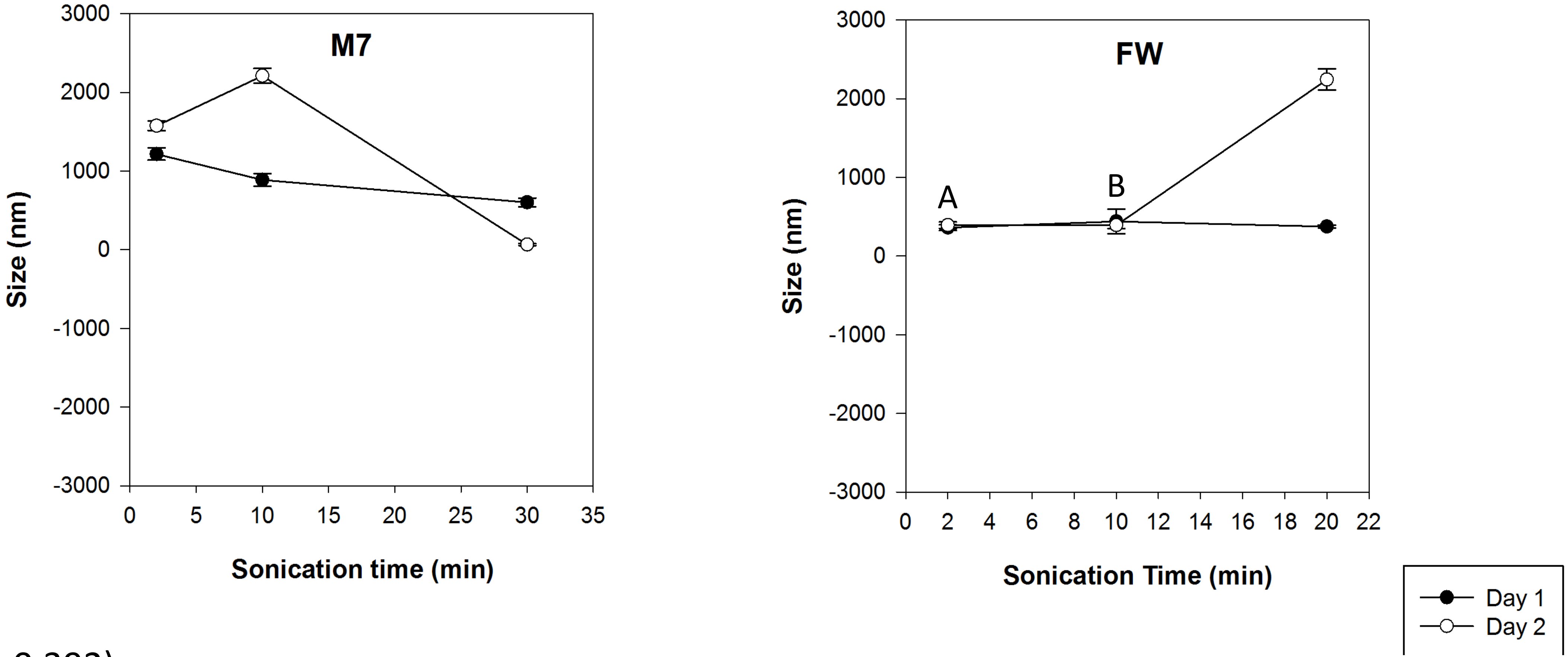


Dynamic Light Scattering (DLS):

- For Day 1 samples, sonicated dispersion medias were placed into DLS to test for agglomerate size

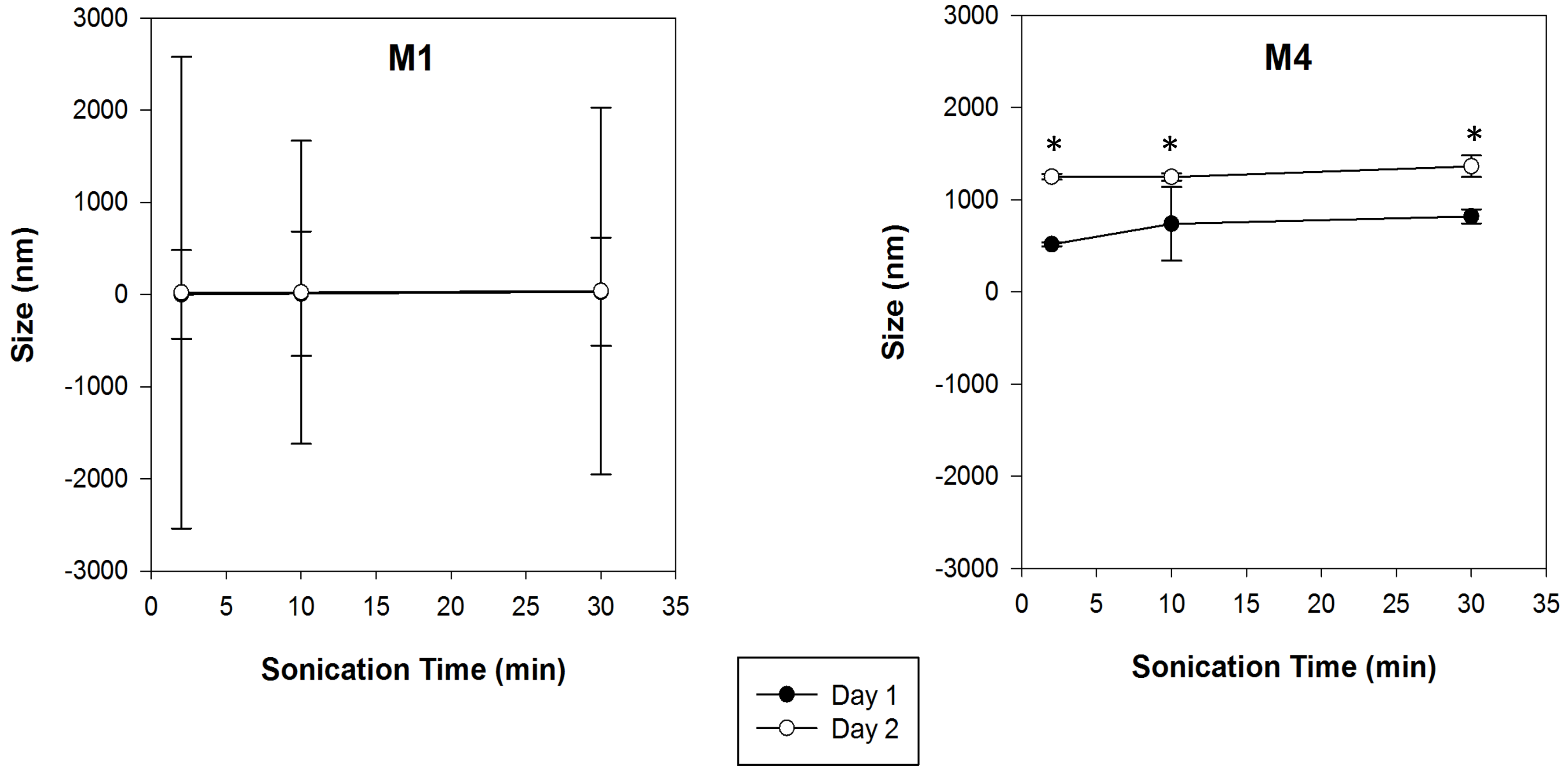
- For Day 2 samples, these same samples were allowed to sit for 48 hours before vortexing sample (no repeat sonication) and measured using DLS

Impact of Ultrasonication on Media 7 and Instant Ocean's Simulated Fresh Water (FW)



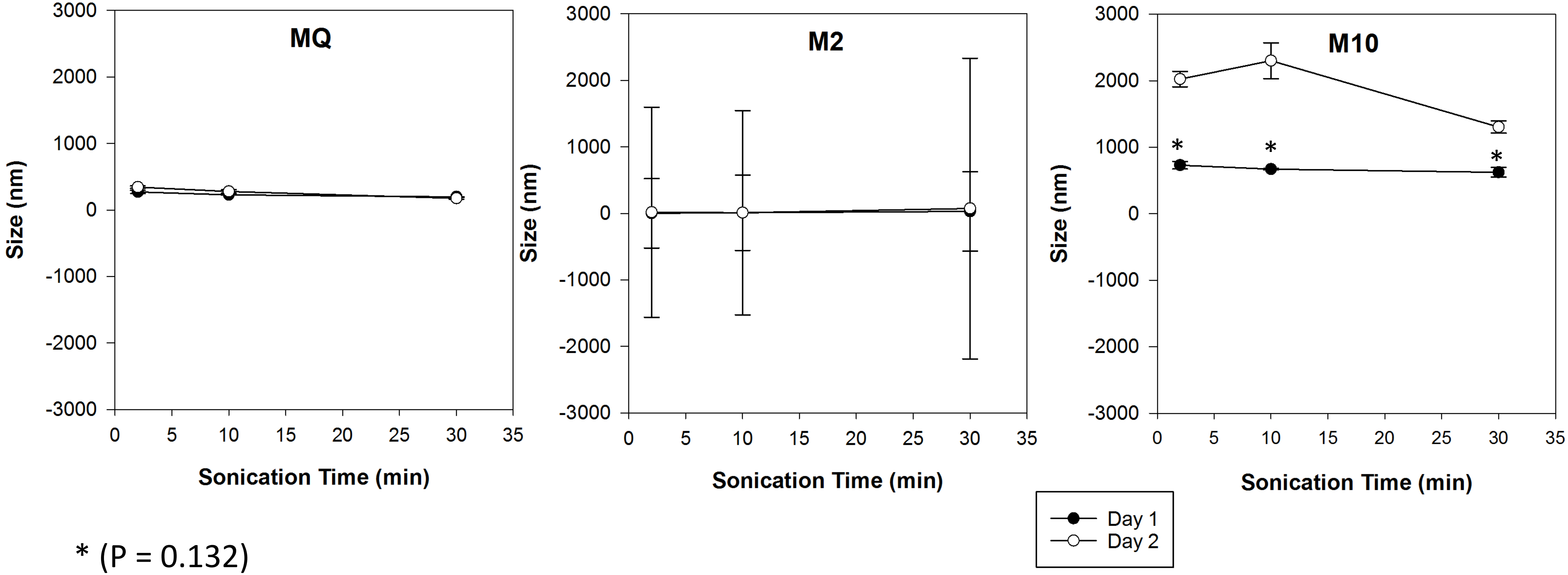
A (P = 0.392)
B (P = 0.642)

Impact of Ultrasonication on Medias 1 & 4



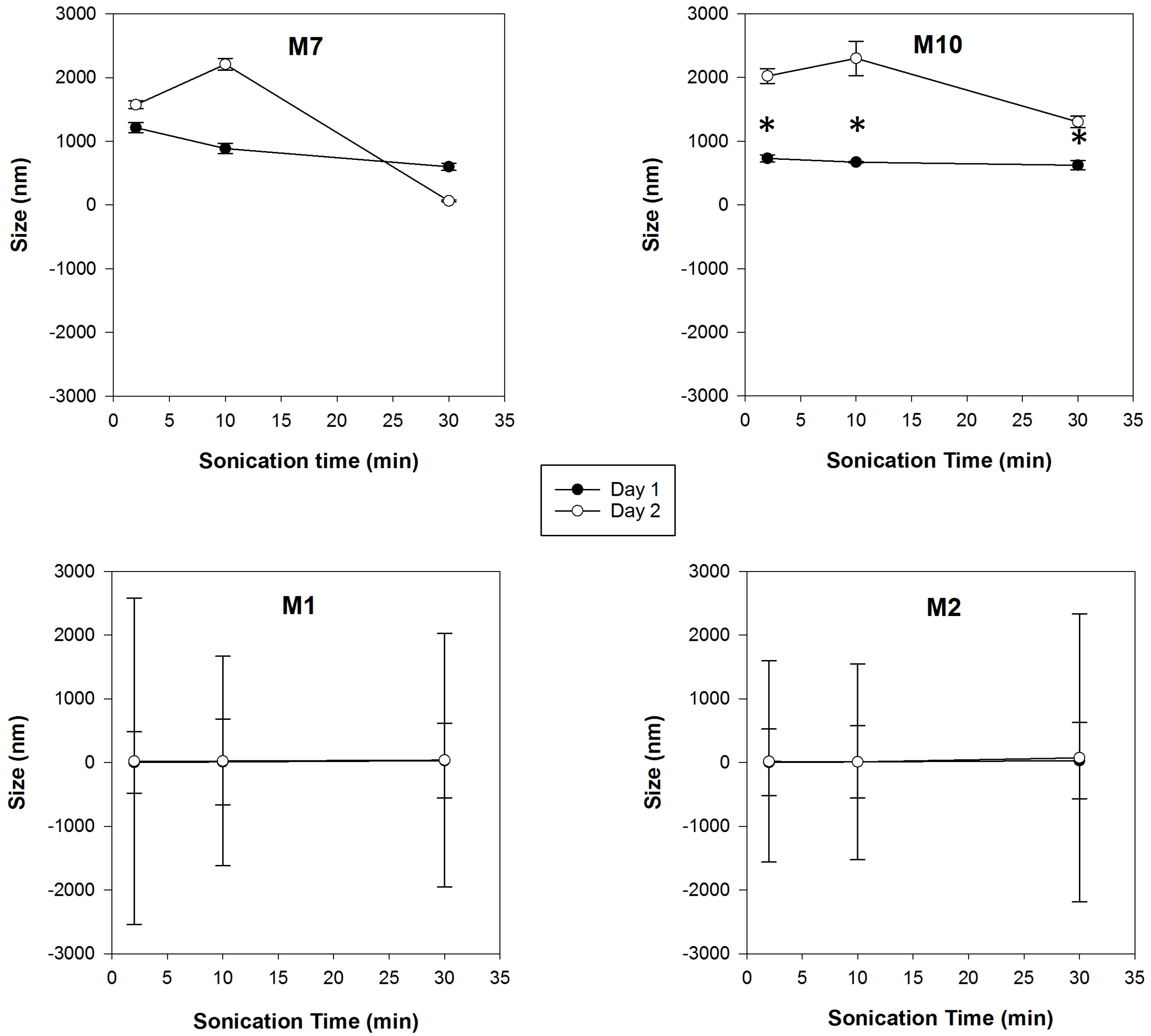
* (P = 0.168)

Impact of Ultrasonication on Medias 2, 10 and Ultrapure (MQ) Water



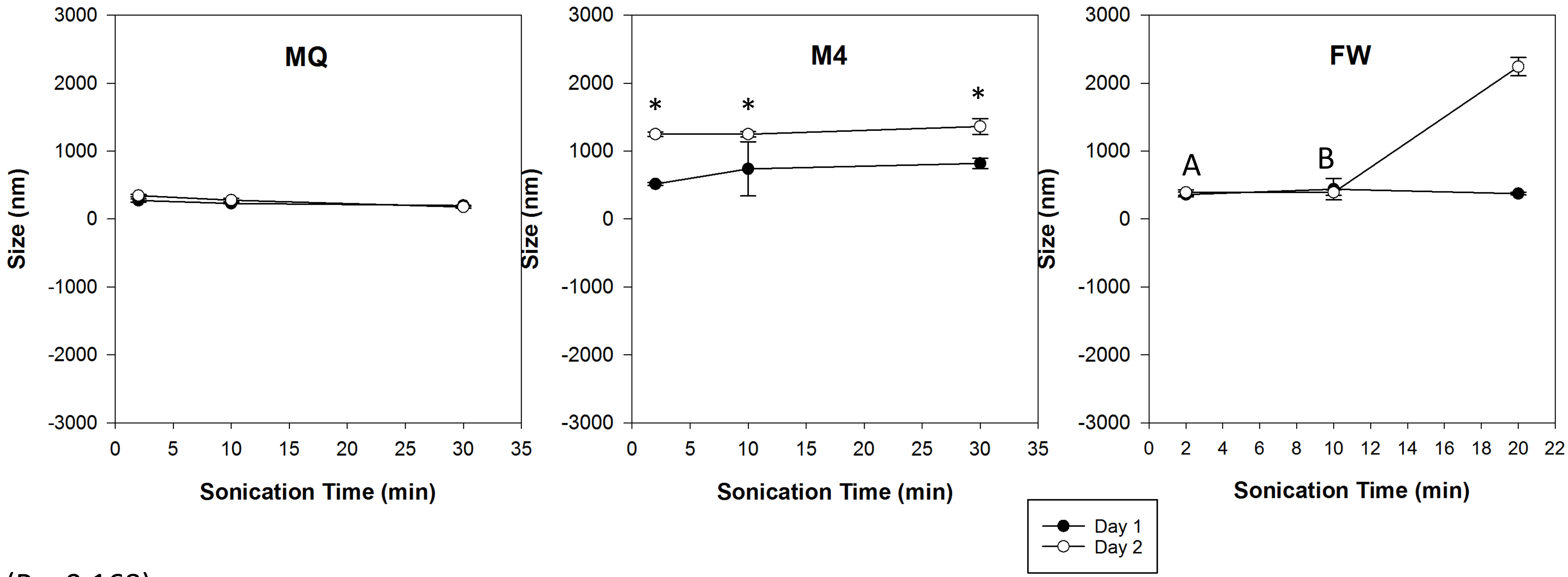
* (P = 0.132)

Impact of Media on Agglomerate Size (HDD)-Discussion



* (P = 0.132)

Impact of Media on Agglomerate Size (HDD) Continued



* (P = 0.168)
A (P = 0.392)
B (P = 0.642)

Fish Water Media- Conclusion

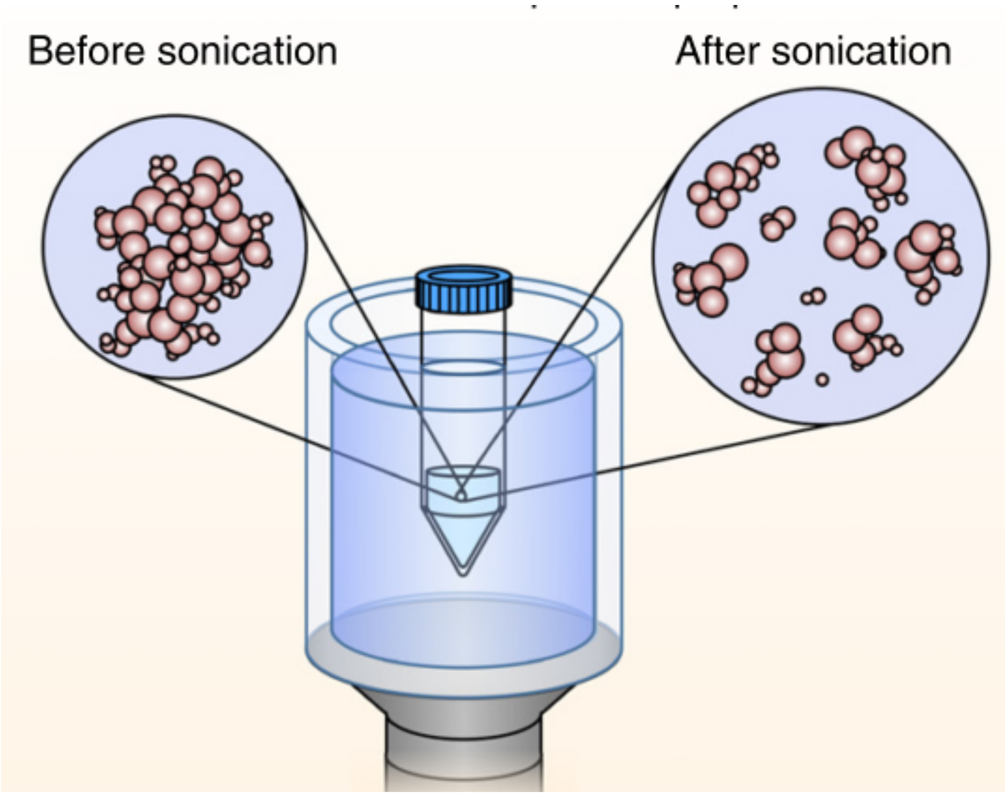
- Media recipe used had an impact on NPs agglomerates ability to deagglomerate during sonication

- We see the medias impact on HDD when comparing 7 different media recipes while holding amplitude, energy and the TiO_2 NPs constant

- Creating a standardized reporting of medias used will increase the chances of a successful reproduction of results between nanotoxicology studies

Sonication Techniques Study- Introduction

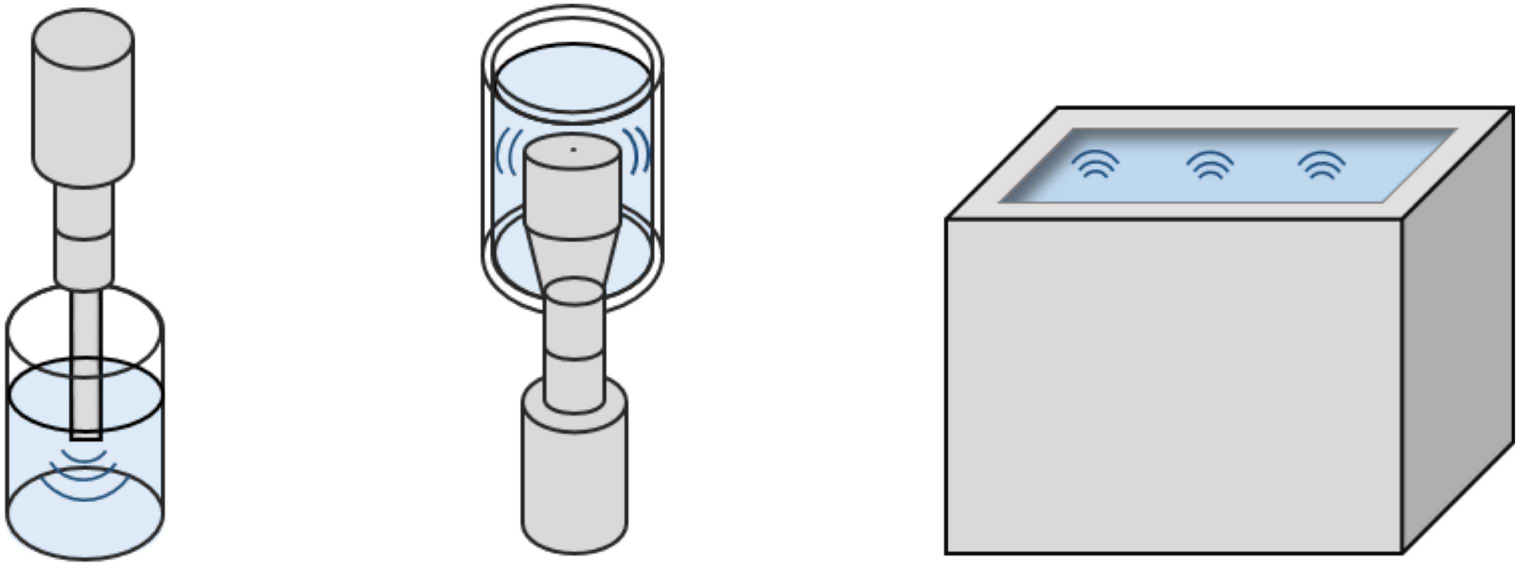
- Issues with reproducibility of results between labs is partly due to lack of standardization in sonication methods
- What is sonication?
  - ➢ Used to disperse and deagglomerate NPs
  - ➢ Technique that utilizes sound energy to agitate and break up agglomerates within a solution to create a stable suspension
  - ➢ Allows for more accurate toxicity testing
- Different sonication methods can impact NPs agglomerates differently



Ultrasonication Systems

Direct Sonicator:
- Probe

Indirect Sonicator:
- Cup horn
- Bath



Probe Cup horn Bath

Amplitude & Calibration of Sonicators

Amplitude:

- The cup horn and probe ultrasonicator utilizes programmed amplitude in the form of a %. Amplitude shows the maximum extent of vibration of probe tip.

- The higher in % the sonicators are set, the greater the intensity and energy available to the sample.

- Amplitude is constant while power itself is varied

Calibration:

- Required by OECD standard protocols.

- Calibration allows the holding of energy equivalents between three different sonication systems.

- Calibrated energy and machine reported power is not equivalent

Standard Protocols

OECD Standard Protocols:

- Preparation of stock concentration of 0.5 – 5.0 x 10^{12} particles/L in ultrapure water at a volume of 125 mL
- Recommends direct sonication (Probe Sonicator) at a power of 40W for 10 minutes

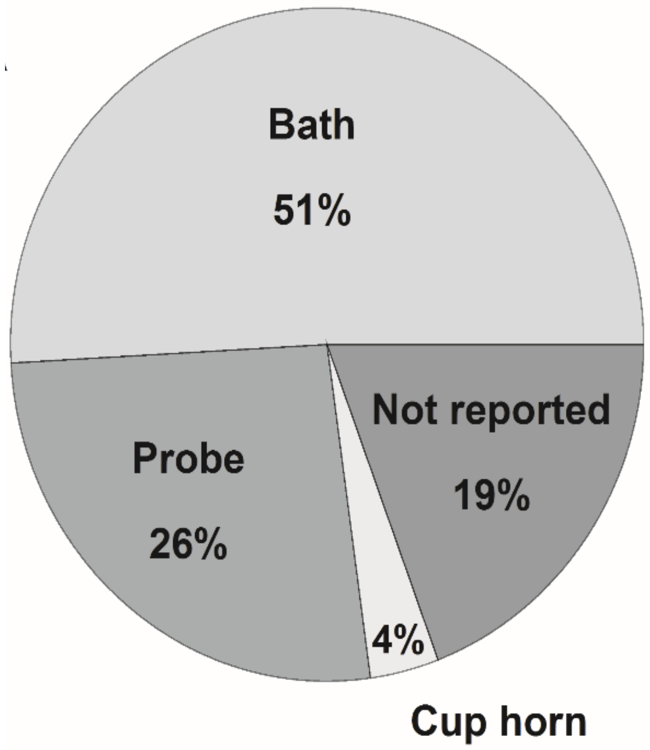
Review of Sonication Practices in Nanotoxicology Studies

- Literature review of 52 nanotoxicology studies to see which sonicators were most utilized

- The majority of studies used bath sonicators

- 19% of studies made no mention of specific sonication system

- Type of sonicator impacts energy intensity



Review of Sonication Practices in Nanotoxicology Studies Continued

Literature Review Findings:

- In over 50% of studies no energy input was reported
- Reported energies ranged across orders of magnitude
- Type of dispersion media used varied and was reported as ultrapure water, buffer solution or appropriate exposure media
- Components of dispersion media such as ions, organic matter and pH/buffers affect agglomeration and overall suspension stability

Sonication Techniques Study- Objectives

- To create a standardized approach to sonication of NPs to better characterize and reduce the current issue with reproducibility of results between nanotoxicology labs.

Methods:

Sonicator Calibration → **NP Stock Preparation** → **Sonication Techniques** → **Dynamic Light Scattering (DLS)**

Sonicator Calibration- Methods

Calibration equation to calculate for actual power (P)

$$P = mC_p \frac{dT}{dt}$$

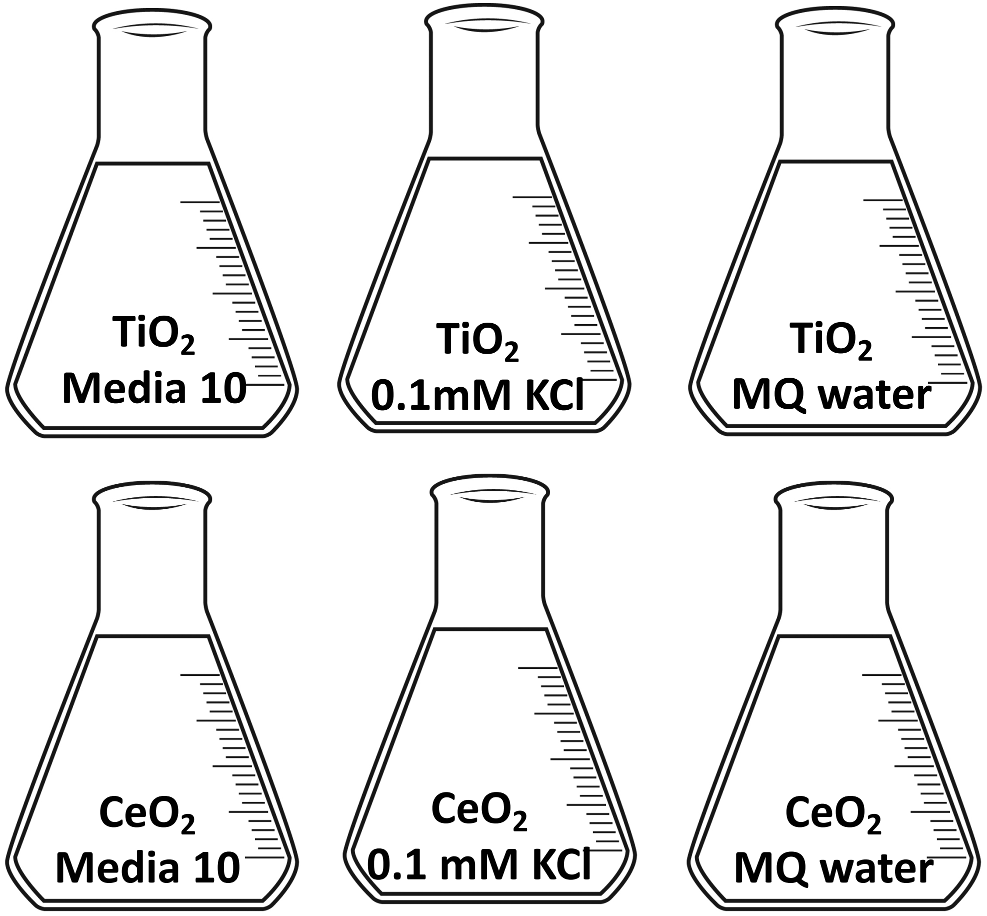
Calculation equation to solve for Sonication time (t)

$$t = \frac{E}{P}$$

NP Stock Preparation, Sonication & DLS- Methods

Method for sample:

- Sonicated with a bath, cup horn and probe sonicator
- Energy was held constant at 8400J
- Another set of samples in MQ water were also run with varied energy 840-84,000J to see energy impact on agglomeration
- DLS was performed directly after sonication to measure HDD

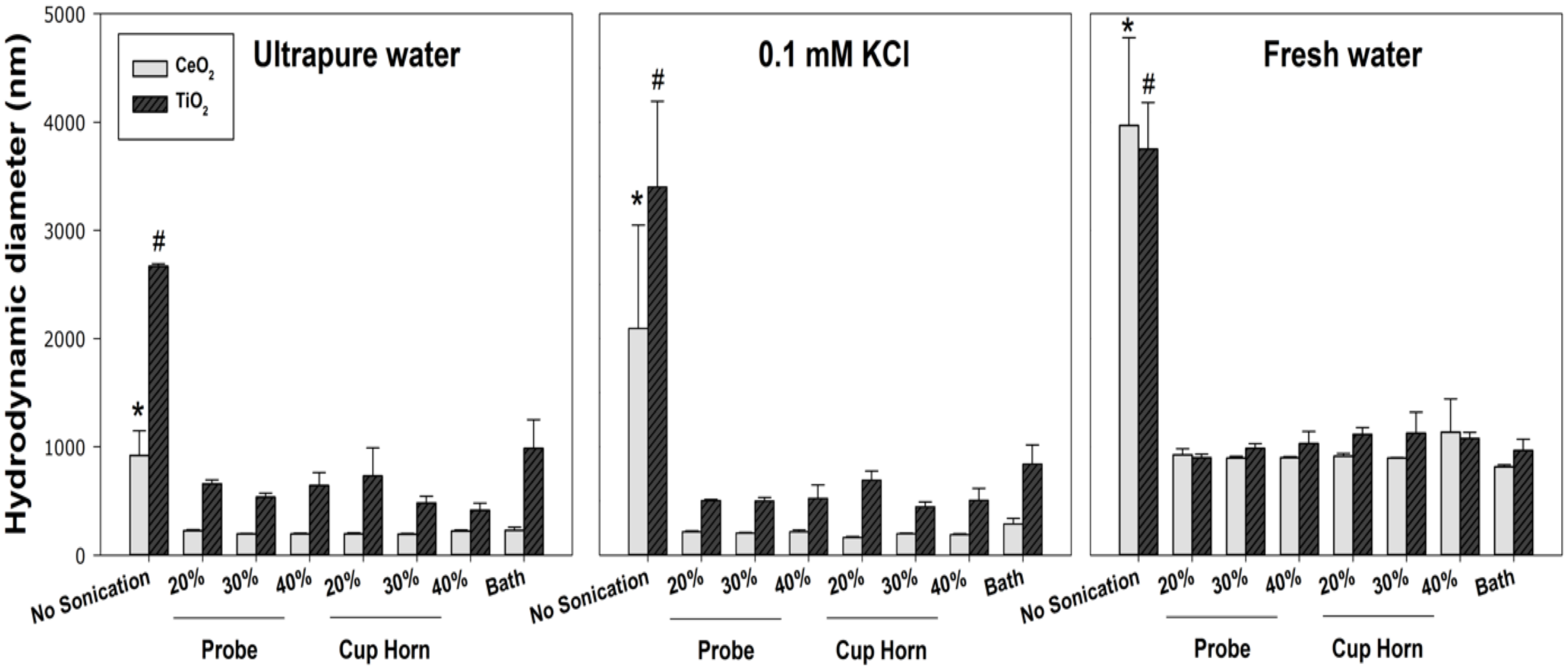


Sonicator Calibration- Results

Programmed Amplitude	Instrument Reported Power (W)	Calibrated Power (W)	Sonication Time (s)
Probe			
20%	33.8	10.0	35
30%	48.8	15.4	23
40%	78.8	19.6	18
Cup Horn			
20%	27.5	22.2	379
30%	52.0	27.9	301
40%	77.3	40.1	209
Bath			
Bath	70	15.0	564

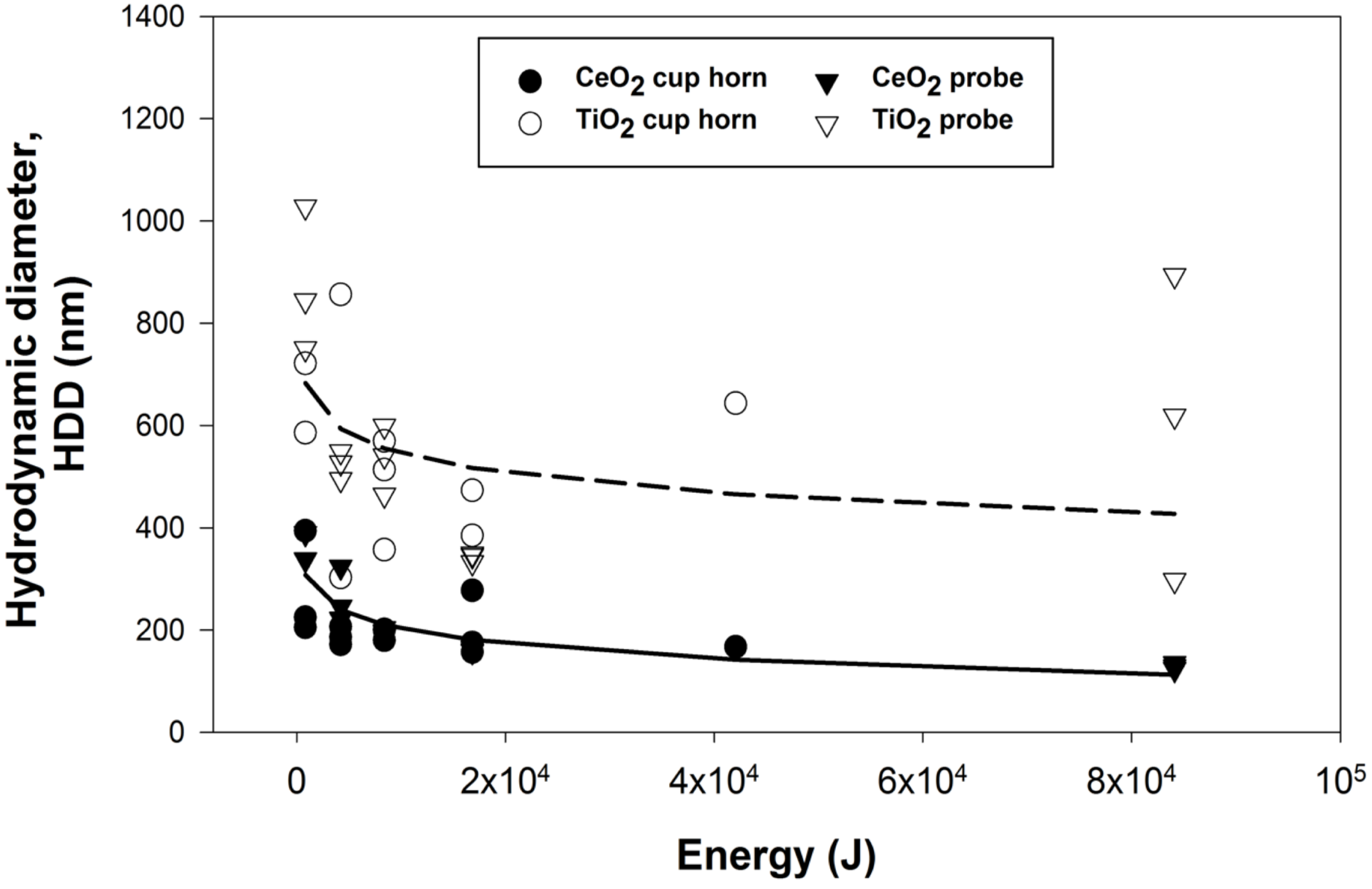
Hydrodynamic Diameter (HDD) and Energy Dependence- Results

- CeO_2 and TiO_2 had a smaller HDD when sonicated compared to no sonication controls
- When energy was held equivalent there was no significant difference in HDD between systems
- TiO_2 was larger than CeO_2 in ultrapure water and 0.1 mM KCl. No significant difference between the two particles in Fresh Water



Hydrodynamic Diameter (HDD) and Energy Dependence Continued

- Energy was varied in order to look at impact of different energy inputs on HDD
- Delivered energy was varied from 840 - 84,000J
- CeO_2 NPs HDD decreased as energy was increased
- TiO_2 NPs HDD decreased until 8400J and from there size increased and varied



Equivalent Energy through Calibration-Discussion

- Aim of this study was to understand the importance of ultrasonication and the reporting of sonication methods in nanotoxicology studies

- Lack of information in published studies included:
  - Sonication system utilized
  - Actual energy input
  - Concentration of NPs within a dispersion media

- Differences in energy input impact NPs agglomerates

- Size of agglomerates can impact a NPs behavior and toxicity during testing

Equivalent Energy through Calibration Continued

- All three sonication systems were calibrated to hold energy equivalent between different systems
- We found that calibrated energy was lower than machine programmed energy in all three sonicators
  - ➢ Due to not accounting for loss of energy as electrical energy is converted to mechanical energy

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Bath			
Bath	70	15.0	564

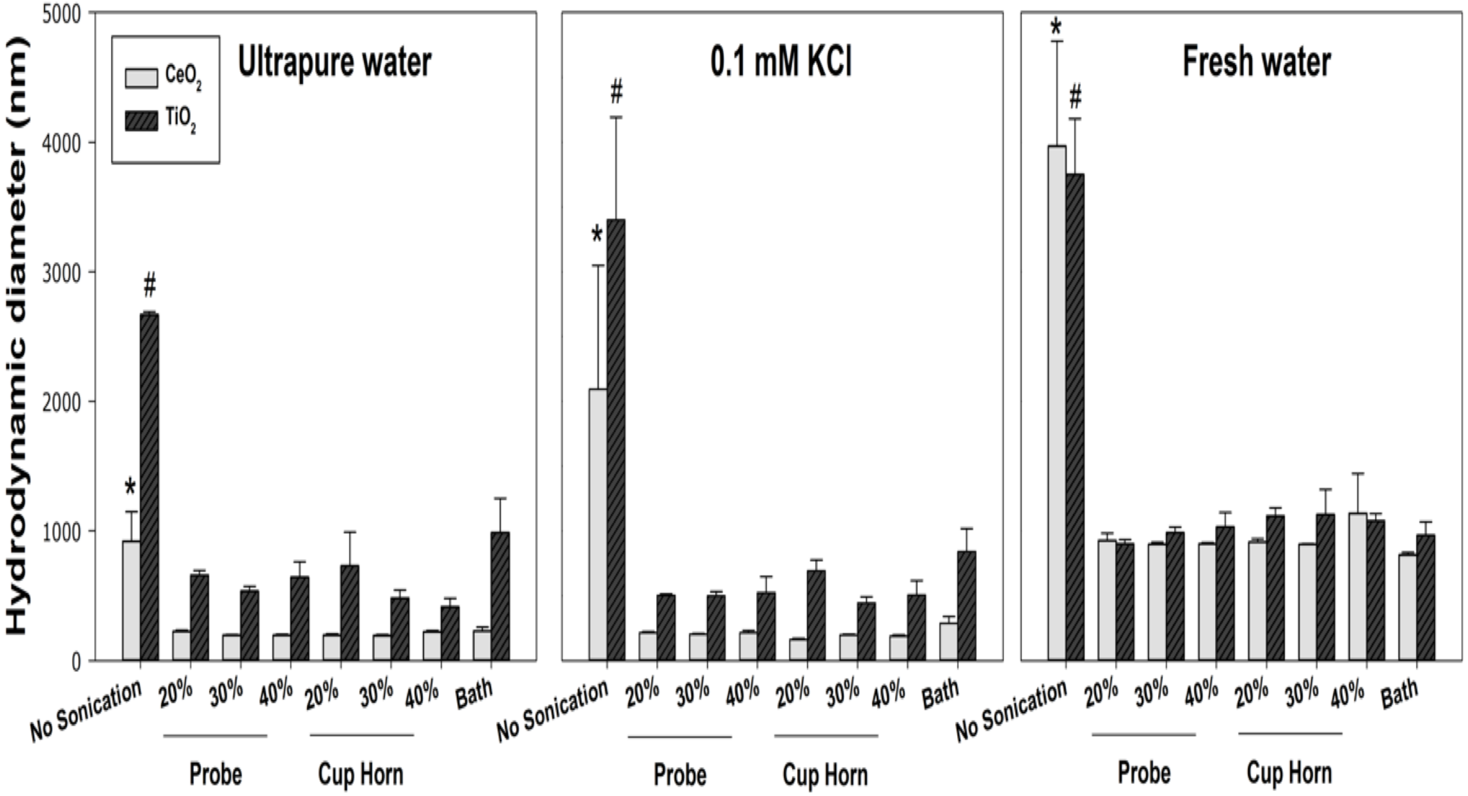
Impact of Ultrasonication on Different Dispersion Medias- Discussion

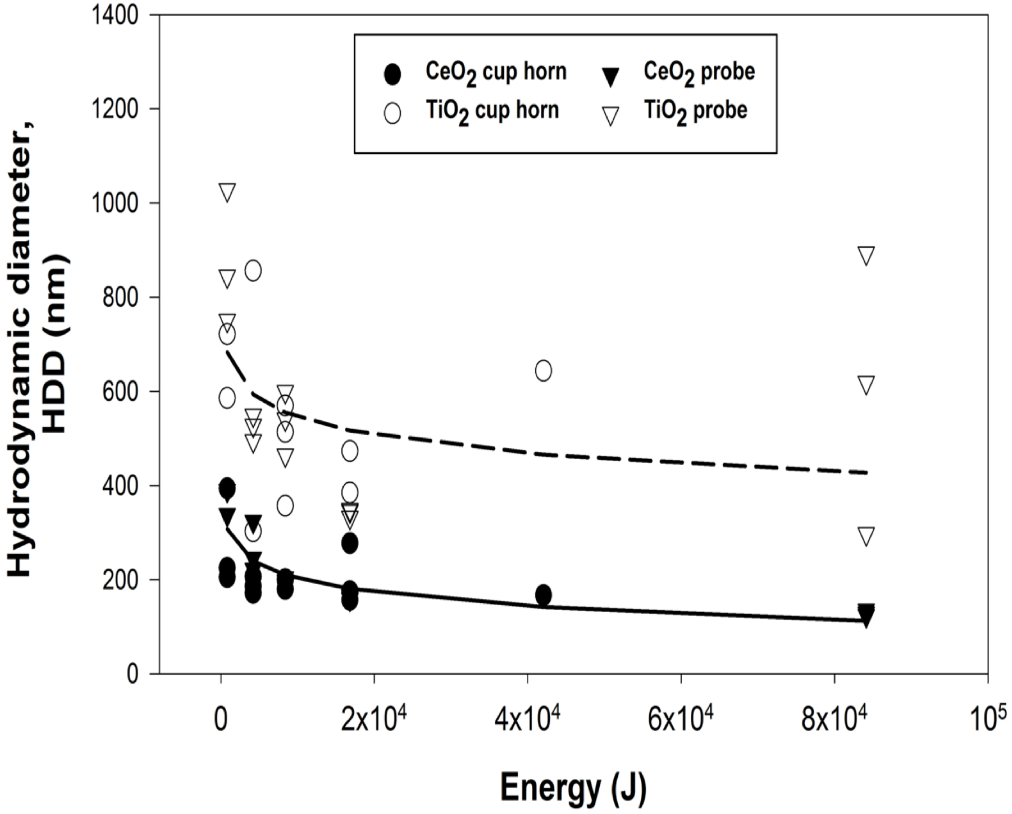
- Additional goals of this study was to look at the impact of ultrasonication on three different dispersion medias
  - 0.1 mM KCl
  - Ultrapure water
  - Simulated fresh water

- Wanted to see if different medias would impact the NPs, TiO_2 and CeO_2, agglomeration behavior

- Wanted to look at energy's impact on NP agglomeration behavior by varying the energy input
  - 840-84,000J

Impact of Ultrasonication on Different Dispersion Medias Continued





Conclusion & Future Studies

Conclusion:

- Many nanotoxicology studies are not following the published recommendations set forth by the OECD on sonication practices

- We demonstrated that sonication is required when creating a stable dispersion media prior to toxicity testing of NPs

- As energy was increased a decrease was seen in NPs agglomerate size with variable results between particles

- When energy is held constant there was no statistical difference between different sonicators used

Future Studies

- We recommend implementing a standardized approach to ultrasonication methods and reporting of calibrated energy in order to increase the reproducibility of results between nanotoxicology studies

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