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**Differences in Engineered Nanoparticle (NP) Surface Physicochemistry Revealed by Investigation of Changes in Copper Bioavailability during Sorption to NPs in the Aqueous Phase**

**Running Head:** Copper sorption onto Nanoparticles in the Aqueous Phase

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**KEYWORDS**

Nanoparticles; sorption; titanium dioxide; copper; zebrafish; *Chlorella vulgaris*

Declarations of interest: none

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## ABSTRACT

Sorption of chemical substances to nanoparticles (NPs) in the aqueous phase strongly influence NP physicochemistry, and investigations of these complex interactions can provide important insight on environmental fate of NPs. The objective of this study was to use differences in copper (Cu) bioavailability to investigate aqueous-phase sorption with NPs of different physicochemistry (Si, perovskite and TiO<sub>2</sub> NPs). Sorption of Cu with NPs was assessed by presence of adsorbent in water and onto NP surface after ultra-centrifugation, and by changes in copper bioavailability under static conditions during exposure of larval zebrafish, and under conditions of continuous agitation during exposure of alga *Chlorella vulgaris*. The presence of TiO<sub>2</sub> NPs reduced total Cu in the water column and Cu bioavailability (measured by growth inhibition, mortality, and metallothionein 2 gene expression) confirming Cu sorption to TiO<sub>2</sub> NPs. Nanoparticle surface area was the most important factor to affect Cu sorption indicated by less bioavailable Cu in presence of smaller TiO<sub>2</sub> NPs. The surface area effect was consistent regardless the exposure conditions (algal continuous agitation or zebrafish static water) and further supported by the lowest total Cu concentration in the water column in the presence of the smallest NP. Results differed with other NP types (i.e. Si NPs) in which Cu sorption was indicated by analytical chemistry, but sorption was not sufficient to significantly alter Cu bioavailability. The bioavailability tests did not indicate Cu sorption with perovskite NPs. Results demonstrate that surface area critically influences sorption, Cu sorption as measured by bioavailability is not affected by agitation or static conditions, and that Cu sorption differs among types of NPs indicating differences among their surface physicochemistry. This article is protected by copyright. All rights reserved

## 1) INTRODUCTION

The lower cost and increasing applications of nanotechnology lead to greater production and release of engineered nanoparticles (NPs) into the aquatic environment. The term engineered NPs is used to identify materials that have all three dimensions in the nanoscale (ISO, 2008), which frequently confers unique physicochemical properties that are attributed to their nanoscale size. A consequence of the increased use of NPs has been the larger release of NPs into the environment, and estimated concentrations of NPs (e.g., TiO<sub>2</sub>-NPs) are at part per million range in some surface waters (Gottschalk et al., 2013; Sun et al., 2014). Within the aqueous phase, some of the unique properties of NPs persist and these properties influence NP environmental fate, interactions with other substances, and potential toxicity. Changes in NP surface physicochemistry can occur in the aqueous phase, including release of ions, sorption of chemical substances to NPs, agglomeration of NPs with other substances and/or particles, and/or changes in NP surface chemistry (e.g., oxidation or changes of coating) (Petersen et al., 2014).

Chemical substances can adsorb to the surface of NPs or absorb within the NP matrix and sorption is dependent on the physicochemistry of both substance and NP. In the aqueous phase, sorption (defined as the taking up and holding of one substance by another (Dabrowski 2001)) can be related to NP surface charge, surface area, the NP matrix, and characteristics of NP agglomerates, among other factors. Based on chemical analyses of NPs, copper, lead and zinc were found to have high affinity for SiO<sub>2</sub> NPs, and sorption was strongly affected by the ionic strength of the solution (Liang et al., 2011). In the aqueous phase, sorption of copper to TiO<sub>2</sub> NPs reduced the total copper concentration in the water column after centrifugation of mixtures (Fan et al., 2016) and the amount of copper adsorbed increased with percentage of

anatase within TiO<sub>2</sub> NPs (Kim et al., 2003). Both anatase and rutile crystalline NP structures adsorbed copper, but the sorption of copper to rutile TiO<sub>2</sub> was four times lower than to anatase TiO<sub>2</sub> NPs (Rosenfeldt et al., 2015).

A challenge for research on NP sorption is that analytical methods applied can disturb the processes under investigation and innovative techniques are required to obtain results that are useful for interpretation. Filtration and centrifugation of NPs to separate NPs from solution are common methods used before application of chemical analysis to investigate sorption and desorption processes (Barakat et al., 2004; Fang et al., 2011; Hu and Shipley 2012). Upon sorption of toxicants (termed here “co-contaminants”) to NPs, bioavailability of the co-contaminants can be enhanced (Henry et al., 2013; Schwab et al., 2013) or reduced (Li et al., 2016; Park et al., 2010), and changes in bioavailability can inform on NP physicochemistry in the aqueous phase (Park et al., 2011). One definition of bioavailability is the proportion of a substance that enters an organism to react with biological molecules (Semple et al., 2004). When substances interact with organisms, molecular responses can be induced and these responses used to indicate changes in substance bioavailability that occur upon sorption with NPs. Aqueous-phase sorption to (C<sub>60</sub>)*n* agglomerates decreased 17  $\alpha$ -ethinyl estradiol (EE2) bioavailability in zebrafish larvae, which was detected by reduction in vitellogenin gene (an EE2 responsive gene) expression (Park et al., 2010). On the contrary, sorption of Hg<sup>2+</sup> to (C<sub>60</sub>)*n* agglomerates led to increased bioavailability of Hg<sup>2+</sup> in zebrafish larvae (Henry et al., 2013). The EE2 was presumed to be absorbed within the (C<sub>60</sub>)*n* agglomerate matrix, whereas more labile sorption of Hg<sup>2+</sup> to (C<sub>60</sub>)*n* agglomerate surfaces was postulated based on the different co-contaminant physicochemistries. The presence of TiO<sub>2</sub> NPs and sorbed As (V) enhanced the As (V) uptake in *Daphnia magna* compared to exposure to As (V) alone. Specifically, double the amount of As (V) was measured by mass spectrometry mainly in *D. magna* gut in the presence of 2 mg Ti /L (equivalent to 3.35 mg/L TiO<sub>2</sub> NPs) and five-times

the amount of As was measured in the presence of increased TiO<sub>2</sub> NPs concentration (20 mg Ti /L or 33.5 mg/L TiO<sub>2</sub>) (Li et al., 2016). Rosenfeldt et al. (2014), observed a 2-fold increase in Ag body burden and 6-fold increase in As body burden in *D. magna* in the presence of 2 mg/L TiO<sub>2</sub> NPs, when compared to exposures to each heavy metal alone. These results with *Daphnia* suggest that TiO<sub>2</sub> NPs acted as a vehicle and increased bioavailability of Ag and As; however, in the same study, the presence of TiO<sub>2</sub> NPs reduced Cu body burden in *Daphnia*, indicating differences in sorption among adsorbates.

Although previous studies have demonstrated sorption of co-contaminants to NPs by detection of changes in substance bioavailability in organisms, this approach has not been used to compare co-contaminant sorption among multiple NPs. The present research aims to increase understanding of co-contaminant sorption onto NPs of different surface physicochemistry and sorption effects directly in the aquatic environment. The analytical techniques chosen are a combination of chemical analyses and environmentally relevant techniques (i.e. investigation of adsorbent bioavailability) that interrogate different aspects of sorption phenomena to examine the effect of NPs with different physicochemistries in the aquatic environment. Our objective was to use copper bioavailability to inform on copper sorption to novel energy-harvesting NPs of different surface physicochemistry: spherical silicon (Si) NPs (Bush et al., 2017), Si NPs with different percentages of boron coating and organo-metal perovskite cell NPs with high potential in photovoltaic industry (Zhu et al., 2015). These were compared to well-studied TiO<sub>2</sub> materials that are estimated as most abundant NPs in surface waters (Sun et al., 2014): TiO<sub>2</sub> bulk (NM100), TiO<sub>2</sub> NM105, TiO<sub>2</sub> NPs of 18 nm diameter and TiO<sub>2</sub> nano-rods of 4-8 nm average size. Two organisms with different exposure conditions, (i.e. direct exposure of green alga *Chlorella vulgaris* to constantly agitated NP suspension compared to indirect - after the sedimentation of the NP agglomerates - static exposure of larval zebrafish *Danio rerio* to NPs) were used to assess

copper bioavailability. Bioavailability was based on examination of the effect of copper on *C. vulgaris* growth and on survival and metallothionein 2 (*mt2*) gene expression in larval zebrafish.

## 2) MATERIALS & METHODS

### 2.1) Chemicals

The following TiO<sub>2</sub> particles were used in the experiments as described: TiO<sub>2</sub> bulk (NM100) of a primary size 200-220 nm and anatase crystal structure, TiO<sub>2</sub> NM105 (JRCNM01005a) with a manufactured size of 30 nm diameter and an anatase:rutile ratio of 85:15; TiO<sub>2</sub> NPs with manufactured size of 18 nm anatase and TiO<sub>2</sub> NPs with manufactured size of 4-8 nm anatase provided by PlasmaChem, Germany (Table 1). The silicon (Si) NPs were composed of monosilane (SiH<sub>4</sub>) with different percentages of diborane (0, 1, 1.5, 2 or 2.5 %) and a primary size of 100 nm; they were provided by the Institute of Energy and Environmental Technology, Duisburg, Germany. Lead iodide perovskite NPs (CH<sub>3</sub>NH<sub>3</sub>PbI<sub>3</sub>) were provided by the École Polytechnique Federal de Lausanne, Switzerland. Aqueous stock suspensions of powdered NPs were prepared in Milli-Q water (grade 18.2 MΩ cm; Millipore, Livingstone, UK) at concentrations of 1-5 g/L. Stock preparations of TiO<sub>2</sub> NPs were sonicated twice for 8 minutes (Jacobsen et al., 2010) and the Si and perovskite NPs were sonicated for 1 min according to recommendations provided by the manufacturers. Determination of average size (hydrodynamic diameter) and surface-related charge (ζ-potential) of NPs in suspension (milli-Q water) was conducted by Dynamic Light Scattering (DLS, Zetasizer Nano Z, Malvern Panalytical, Malvern, UK) at a concentration of 2 mg/L. The co-contaminant used was CuSO<sub>4</sub>·5H<sub>2</sub>O and purchased from Sigma-Aldrich (lot#: SLB4752V) with purity >98.0%.

## 2.2) Analytical chemistry

Aqueous phase copper concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500ce, School of Chemistry, University of Edinburgh) to investigate sorption of Cu to NPs and the effect of NP surface area. For the analytical chemistry analyses representative NPs were chosen based on the results of the concentration-response assays (section 2.4). A single nominal concentration of Cu salt that was in the range of the concentrations used for the following zebrafish larvae concentration-response assays (0.25 mg/L) that was added to different concentrations (0, 2, 4, 8 and 16 mg/L) of Si NPs, NM105, TiO<sub>2</sub> (18 nm) or TiO<sub>2</sub> (4-8 nm) NPs. The preparations (20 mL) were stirred with a magnetic bar for 24 h for the sorption equilibrium to occur (Engates and Shipley 2011; Rashidi et al., 2010) and then centrifuged at 24,000 rpm for 1 h polycarbonate tubes (JA-25.15 rotor, Beckman Coulter UK Ltd) (Malleve et al., 2014). The supernatant (10 ml) was collected and digested with 15% nitric acid (trace metal grade) for measurement of total Cu in the water column. From the remaining portion (10 mL) of each preparation, 9.9 ml were carefully removed and the remainder (0.1 mL, containing the pellet) was mixed by pipetting, allowed to dry, and used to investigate presence of Cu on the surface of NPs. Chemical analyses on the surface of the NPs in the pellet samples were conducted by X-ray photoelectron spectroscopy (XPS), Nexus, Newcastle University, as it offers minimal manipulation of the samples and insight on the surface composition.

## 2.3) Test organisms

*Chlorella vulgaris* (Culture Collection of Algae and Protozoa 211/12, originally obtained from Scottish Marine Institute, Oban, UK) culture was grown in OECD TG 201 medium (OECD 2011) in 250-mL Erlenmeyer flasks (Scientific Laboratory Supplies, Coatbridge, UK) under constant rotary agitation (225 rpm), illumination (120  $\mu\text{mol}/\text{m}^2\text{s}$ ) and temperature



(23 °C) based on our established methods (Kalman et al., 2015). When the cell density reached  $\sim 10^6$  cells /mL, the stock culture was maintained under static conditions (illumination of 50  $\mu\text{mol}/\text{m}^2\text{s}$ ) in a 16:8 h light:dark photoperiod at 20 °C. Cultures were maintained by transferring a small aliquot into fresh sterile medium and were checked every two months for bacterial contamination by plating on nutrient agar (Oxoid Ltd, Basingstoke, UK).

Zebrafish (*Danio rerio*) were obtained from the zebrafish research facility (Heriot-Watt University), and husbandry, spawning and experimentation were conducted with complete approval and according to animal welfare regulations of the UK Home Office. Water for experiments was synthesised fresh water with characteristics consistent with the OECD guidelines for fish embryo toxicity tests (OCDE 2013). Characteristics of water chemistry were 79, 12, 17 and 2 mg/L of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$ , respectively; pH of 7.7; temperature was maintained between 28-30°C, and a 12-h photoperiod. Hatching occurred between 48 and 72 hours post fertilization (hpf), and fish of age 72 hpf were used for the 96-h concentration-mortality assays.

#### 2.4) Experimental design

##### 2.4.1) Concentration-response assays

Preliminary experiments were performed to establish concentration-response (growth inhibition) relationships for *C. vulgaris* to aqueous Cu (as  $\text{CuSO}_4$ ). The toxicities of Si NPs, perovskite NPs and  $\text{TiO}_2$  NPs, and bulk  $\text{TiO}_2$  to algae were individually tested up to concentration of 100 mg/L (no effects on algal growth inhibition or larvae survival were observed). The NPs selected for the following study were dispersed in milli-Q water (as described in the *Chemicals* section), bath sonicated and the suspensions were vortexed before the preparation of each treatment to ensure homogeneous solutions.

Algal growth inhibition assays were performed in 250-mL Erlenmeyer flasks according to the OECD test guideline (OECD 2011) or in plastic 24-well plates using *C. vulgaris* in the exponential growth phase. Temperature and light conditions for toxicity tests were identical to those used for culture growth. The Cu concentration-growth inhibition experiments were carried out in triplicate using five concentrations (0, 0.01, 0.02, 0.025, 0.03 and 0.04 mg/L) of Cu. The initial concentration of the inoculum was  $10^4$  cells per mL, which was required to ensure that cultures were at exponential growth. Cell density was determined after 24, 48 and 72 hours of exposure by measuring *in vitro* fluorescence of acetone-extracted chlorophyll *a* (Kalman et al., 2015) in experiments carried out in Erlenmeyer flasks (Cu, nano and bulk TiO<sub>2</sub>). In the case of Si and perovskite NPs acute toxicity tests, 1.5 mL of each test suspension were added in triplicate to wells of 24-well plates due to limited amount of Si and perovskite NPs available. Plates were sealed with parafilm to avoid evaporation. *In vivo* chlorophyll *a* was measured by fluorescent plate reader (Molecular Devices SpectraMax M5) using excitation wavelength at 435 nm and emission wavelength at 685 nm at the same time points. The NP exposure concentrations ranged between 1 and 100 mg/L for Si and perovskite NPs, and the TiO<sub>2</sub> NPs and bulk TiO<sub>2</sub>. Copper sorption on NPs was assessed by investigating changes in algae growth inhibition by exposing *C. vulgaris* to various concentrations of Cu in absence and presence of NPs. Two parallel Cu concentrations in a range of 0 to 60 µg/L were prepared in Erlenmeyer flasks. In one Cu dilution series, 2 mg/L of NPs were added. The parallel dilutions experiment was conducted once for each NP type. Algal growth inhibition, as chlorophyll fluorescence, was determined after 72 h of exposure. The bioavailability experiments are presented in detail in Table 2.

Zebrafish larvae age 72-hours post fertilization (hpf) were exposed to Cu to establish the LC<sub>50</sub> and sub-lethal concentrations over 96-h exposure period. All the Cu exposures (0-0.36 mg/L) were conducted in glass beakers containing 50 ml of fish fresh water medium, and 20-

30 larvae were exposed in each beaker. For each experiment, control beakers were included (one of fish water and one of fish water and NPs). Acute (96 h) toxicity tests were conducted to determine lethal effects of NPs in larvae aged 72-168 hpf (Table 2). Larvae were exposed to NP concentrations between 0 and 200 mg/L, and 10 larvae were individually exposed to each concentration in 96-well plates with an exposure volume of 200  $\mu$ L. The 96-well plates were used for the NP acute toxicity assessments because of the limited amount of Si and perovskites NPs available. For the zebrafish co-contaminant experiments, two parallel dilutions of Cu were prepared (0-0.36 mg/L) in glass beakers and 2 mg/L of NPs were added to one dilution series. The Cu dilution series with added NPs were stirred with a magnetic stir bar at medium speed for 24 h prior to fish exposure. Every experiment had 20-30 larvae per preparation with a final volume of 50 mL in glass beakers. The parallel dilutions experiment was conducted once for each NP type and four times for NM105 using four different NP concentrations added in copper dilution series (1, 2, 3 and 4 mg/L). The mortality of the larvae was recorded daily, pH did not drop under 6.5 and the dead larvae were removed from the preparations.

#### 2.4.2) *mt2* gene expression

To evaluate the concentration-response relationship between Cu concentration and *mt2* gene expression, fish were exposed (24 h) to Cu (0, 0.12, 0.24 and 0.36 mg/L) or Cu and 2 mg/L of NPs (Si NPs, TiO<sub>2</sub> 18 nm or TiO<sub>2</sub> 4-8 nm) as shown in Table 2. Three replicates were investigated for each condition. Larvae of 96hpf were sampled and stored at -80 °C until further analysis. Total RNA was extracted from larvae 96 hpf (30 larvae per replicate) after a 24-h exposure period, using RNeasy MiniKit for animal tissue, Qiagen, West Crawley, UK as previously described (Boran et al., 2016). Briefly, zebrafish larvae were homogenized with a pestle, a DNase treatment (Qiagen, West Crawley, UK) was included as a step during the

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extraction to prevent sample contamination from DNA, the RNA was eluted in 30  $\mu$ L of sterile RNase/DNase free water and stored at -20  $^{\circ}$ C until further analyses. The concentration of the total RNA was measured using a NanoDrop Spectrophotometer (ND-2000). Pure RNA (i.e. no or minimal protein and phenol contamination) was ensured for all samples used for further analysis with 260/280 ratio between 2.0-2.2 and RNA was diluted to a final concentration of 100 ng/ $\mu$ L. The cDNA was synthesised using Precision nanoScript2 kit by PrimerDesign (Eastleigh, UK) with the following conditions: annealing at 65  $^{\circ}$ C for 5 minutes, extension at 42  $^{\circ}$ C for 20 minutes and inactivation of the reaction for 10 minutes at 75  $^{\circ}$ C. The cDNA was stored at -20  $^{\circ}$ C until gene expression analysis.

Primers designed for zebrafish from Primer BLAST (NCBI) for the following genes: *mt2* (NCBI Reference Sequence: NM\_001131053.2, Forward (5'-3'): TGTTCTCAATCTTGTCTGTTTAATG, Reverse (5'-3'): TGTAGAGCACTATCAGAATAAACG, with product length of 108 bp and annealing temperature at 60 $^{\circ}$ C) and  *$\beta$ -actin* (NCBI Reference Sequence: NM\_131031.1, Forward (5'-3'): ACACAGCCATGGATGAGGAAATCG, Reverse (5'-3'): TCACTCCCTGATGTCTGGGTCGT, with product length of 138 bp and annealing temperature at 60 $^{\circ}$ C) (Boran et al., 2016). Precision PLUS Mastermix with SYBRGreen, PrimerDesign (Eastleigh, UK), was used for the quantitative PCR. Fluorescence was detected after 40 two-step cycles (denaturing temperature at 95  $^{\circ}$ C, primer specific annealing temperature and extension step 60  $^{\circ}$ C) by StepOne Real Time System (Applied Biosystems, Warrington, UK). The efficiency of the qPCR was calculated ( $e = 10^{(-\frac{1}{slope})} - 1$ ) based on a 4-point standard curve, and an efficiency of between 0.9 and 1.1 was required for further analyses. The threshold cycle  $C_T$  measured by the RT qPCR determined the expression levels of the housekeeping gene ( *$\beta$ -actin*) and *mt2*. The *mt2* levels were then normalised after the  *$\beta$ -actin* levels ( $\Delta C_T$ ) and the differences between the experimental control group and the

exposed larvae were obtained by the  $\Delta\Delta C_T$  method. The fold changes ( $2^{\Delta\Delta C_T}$ ) of *mt2* levels in exposed samples were compared to the average  $\Delta\Delta C_T$  of the control larvae (Henry et al., 2013).

### 2.5) Statistical analyses

All statistical analyses were conducted using *R statistics* (Team 2015). The algal growth inhibition and Cu concentration relationship, in absence or presence of NPs, were modelled using the four-parameter log-logistic function and compared using *anova* in ‘drc’ package in R. Zebrafish mortality was modelled by logistic regression with the independent variables copper concentration, treatment (presence or absence of NP), and concentration and treatment interaction. The logistic regression models were generated by iterative maximization of the likelihood function, and independent variables and their interaction were included if they significantly improved the model (likelihood ratio test, based on Wald  $\chi^2$  distribution). The median lethal concentration (LC50) was calculated by the logistic regression model and the 95% confidence interval (CI) was calculated using the *logit* model in R statistics. For the gene expression analyses, the relative fold change was modelled by general linear model (GLM) according to the independent variable of Cu concentration (p-value of <0.05). Differences in fish mortality among treatments (i.e., presence or absence of NPs) were determined by pair-wise contrast statements with a p-value of <0.05.

## 3) RESULTS

### 3.1) NP Characterisation and Chemical Analyses

All NPs tested tended to agglomerate in milli-Q water with average agglomerate diameter between 314 and 637 nm (NP concentrations 2 mg/L), which was a higher average size than

reported by manufacturers (Table 1). The average agglomerate size of Si NPs ranged between 324 and 627 nm diameter, with higher average agglomerate size observed in Si NPs with B coating of 1.5% and the size decreased in Si NPs with B 2% and B 2.5% coating. For all TiO<sub>2</sub> NPs measured in the present study,  $\zeta$ -potentials were between -30 and 30 mV. The smallest TiO<sub>2</sub> NPs (4-8 nm) showed  $\zeta$ -potential very close to zero (-2 mV) and a negative  $\zeta$ -potential and high agglomeration was observed in perovskite NPs and the Si NPs were found more stable with higher absolute value of  $\zeta$ -potential.

Sorption of copper to NPs was indicated by changes aqueous copper concentration and detection of copper in pelleted NPs. Specifically, total copper concentration decreased by 4.7 %, 1 % and 59 % when 2 mg/L of Si NPs, NM105 and TiO<sub>2</sub> 4-8 nm NPs were present, respectively. Total copper reduced further with increasing concentration of all NPs (Figure 1A); however, at the same NP concentration, more copper was removed by the smallest TiO<sub>2</sub> NPs (4-8 nm). Copper was detected in the NP pellets in all preparations after XPS analyses (Supplementary Data).

### 3.2) Concentration-response assays

There was no effect of NP exposure (without copper) on *C. vulgaris* growth or zebrafish survival at the concentrations tested in the sorption studies. No larval zebrafish died after exposure to 2 mg/L of TiO<sub>2</sub> NPs or bulk TiO<sub>2</sub>. No mortality was found in the zebrafish larvae control treatments and *C. vulgaris* controls met the growth targets. No larval mortality was observed after 2 mg/L exposures of perovskite, Si and Si-boron NPs and no growth inhibition was found in *C. vulgaris* after exposure to Si and Si-boron NPs (data not shown). For the perovskite NP (CH<sub>3</sub>NH<sub>3</sub>PbI<sub>3</sub>) the EC<sub>50</sub> value for *C. vulgaris* was estimated at 25.002 mg/L (Supplementary Data) and 2 mg/L was considered a no-effect concentration. This is the first study to assess sorption of aqueous copper to Si and perovskite NPs as well as evaluate

toxicities of Si, boron coated Si NPs and lead iodide perovskite NPs to *C. vulgaris* and zebrafish larvae.

No differences in the inhibition of *C. vulgaris* growth or in larval zebrafish mortality were observed after copper exposure in the presence of Si or perovskite NPs, and copper toxicity was reduced in the presence of all TiO<sub>2</sub> NPs. Both growth inhibition of *C. vulgaris* after 72 h and zebrafish mortality (96-h exposure) increased significantly with copper concentration ( $p < 0.001$ ), as expected. The presence of Si NPs or perovskite NPs did not significantly affect copper toxicity in algae or zebrafish larvae (Supplementary Data), therefore only Si NPs without B coating were selected for the chemical analyses and the gene expression study. Significant reduction in algal growth inhibition and larval mortality were observed in the presence of TiO<sub>2</sub> NPs and bulk ( $p < 0.001$ ) (Figure 2 and 3). Reduced inhibition of algal growth and reduced larval mortality were most evident in the case of TiO<sub>2</sub> NPs with smallest particle size. Increased sorption occurred with greater concentration of NPs present in the copper dilution series. Specifically, increasing NM105 concentration decreased copper toxicity in larval zebrafish as indicated by the difference in 96-h LC50 ( $\Delta$  LC50) between copper alone and copper/NM105 mixtures concentration – response curves (Figure 4).

### 3.3) *mt2* gene expression

Induction of *mt2* occurred with increased copper concentration (GLM,  $p < 0.001$ ) in zebrafish larvae, and *mt2* expression increased up to 8-fold after 24-h exposure to 360  $\mu\text{g/L}$  of copper relative to unexposed control fish. The presence of Si and TiO<sub>2</sub> 18nm NPs did not reduce the expression of *mt2*; however, *mt2* expression decreased significantly when zebrafish larvae were exposed to copper in the presence of the TiO<sub>2</sub> 4-8 nm, the smallest particle tested (GLM,  $p < 0.01$ ,  $R^2 = 0.79$ ) (Figure 5). For the *mt2* expression investigation, representative NPs

were chosen based on the results of the concentration-response assays (section 2.4). Exposure to NPs alone did not induce *mt2* expression.

#### 4) **DISCUSSION**

All NPs tended to agglomerate in the aqueous phase and this evidence was supported by both measurements of the average agglomerate size in suspension and  $\zeta$ -potential. In the present study, all NPs tested formed visible agglomerates that settled on the bottom of the static exposure beakers within 24 h of the start of zebrafish larvae exposures and is in agreement with the measurements of the hydrodynamic diameter were conducted in milliQ water. No changes were observed in Si NPs morphology or particle agglomeration after B doping (Ge et al., 2013) and agglomeration and precipitation of lead halide perovskite particles have been enhanced with time and exposure to excitation source (Gomez et al., 2017). Agglomeration of TiO<sub>2</sub> NPs, with average hydrodynamic diameter between 1024 to 1792 nm, has been reported previously to occur within 10 minutes after the introduction of NPs in fresh water media such as algae medium (OECD 2011), hard-water medium and *Lumbriculus variegatus* medium (Nur et al., 2015; OECD 2007). The absolute value of  $\zeta$ -potentials for all TiO<sub>2</sub> NPs was found to be between 30 mV and -30mV, a measurement that is consistent with other studies and has been attributed to the lack of sufficient repulsive forces between NPs to keep them in suspension in the aqueous phase (Honary and Zahir, 2013; Nur et al., 2015). The  $\zeta$ -potential of the smallest TiO<sub>2</sub> NPs (4-8 nm) suspensions was very close to zero, which is in agreement with the higher agglomerate sizes observed among TiO<sub>2</sub> NPs (Berg et al., 2009).

In the presence of the smallest TiO<sub>2</sub> NPs (4-8 nm), less Cu was measured in the water column, compared to NM105 and Si NPs at the same concentrations, indicating that more Cu was adsorbed onto NPs likely because of greater surface area available for copper sorption.



Previous studies have observed increasing heavy metal adsorption proportionally to higher surface area after increasing concentration of adsorbent. Specifically, Cu, lead and cadmium (Cd) adsorption increased with increased concentration of modified chitosan microspheres (Ren et al., 2013) and Cd and zinc adsorption increased in a linear manner with increasing concentration of magnetic hydroxyapatite NPs (in concentrations <100 mg/L of NPs) (Feng et al., 2010). Therefore, there is evidence for linear relationship of adsorbent concentration and surface area available for adsorption. Both studies showed that adsorption after a critical concentration of adsorbent, reached a plateau or started to decrease. Based on adsorbate mass and adsorbent concentration proportional linear relationship, the sorption of Cu did not increase as it would be expected according to the increasing surface area in the present study (i.e. dotted line in Figure 1B). Furthermore, the total Cu adsorbed reached a plateau instead of increasing (Figure 1B), that can be explained by agglomeration of NPs in suspensions that would consequently lead to a decrease in surface area available for interaction with Cu. As discussed earlier, the smallest TiO<sub>2</sub> NPs showed the highest average agglomerate size in aqueous suspension and as shown in Figure 1A, Cu adsorption is reaching a plateau with higher concentration of TiO<sub>2</sub> NPs (4-8 nm) present in suspension. The DLS analyses reported agglomeration of all NPs in the aqueous phase, however, the average agglomerate size (Table 1) cannot inform on the most abundant particle size in suspension. Therefore, corresponding surface area in Figure 1B was calculated by the reported manufactured particles dimensions. Agglomeration has been reported to affect the sorption capacity of TiO<sub>2</sub> NPs at high concentrations, such as 150 mg/L (Li et al., 2010), but no effect was observed in concentrations of lower range (0.2-2 mg/L) (Farkas et al., 2015). The results of the present study further support that sorption capacity of NPs depends on the different physicochemical properties of the NPs (i.e. different material, surface area or shape), while agglomeration of NPs can decrease their sorption capacity.

The NPs used in the present study to evaluate Cu sorption, did not cause any toxicity in the organisms at the concentration selected to assess Cu sorption. Exposure to TiO<sub>2</sub> NPs or bulk TiO<sub>2</sub> at tested concentrations (2 mg/L) did not affect growth of algae compared to unexposed controls, which is consistent with previous research (Hartmann et al., 2010). No larval zebrafish died after exposure to 2 mg/L of TiO<sub>2</sub> NPs or bulk TiO<sub>2</sub>, which is consistent with previous studies of TiO<sub>2</sub> NPs toxicology (Boran et al., 2016; Boyle et al., 2015). Exposure of zebrafish to TiO<sub>2</sub> for 24 h did not cause any induction of *mt2*, which is in accordance with previous investigation of *mt2* gene expression in zebrafish after exposure to TiO<sub>2</sub> NPs (Boran et al., 2016; Park and Yeo 2013; Tan and Wang 2014). It has been observed that the organo-metal halide cells in the aqueous phase can release the metal halide (e.g. PbI<sub>2</sub>) and cause mortality in zebrafish larvae at high concentrations (Babayigit et al., 2016; Benmessaoud et al., 2016), however, the low perovskite concentrations tested in the present study did not cause toxicity to *C. vulgaris* or zebrafish larvae.

The changes in Cu bioavailability in the presence of NPs suggest sorption of Cu onto NPs present in suspension. Si and perovskite NPs did not alter Cu toxicity in algae or zebrafish larvae, suggesting that the interactions between copper and Si or perovskite NPs that did not alter copper bioavailability (and therefore toxicity) in algae or zebrafish. The 96-h zebrafish concentration-response curves suggest that not enough copper was adsorbed on Si NPs to reduce copper bioavailability, while the actual measurement of copper in the presence of NPs suggests that sorption of copper does occur on the surface of 2 mg/L Si NPs after 24 h of mechanical stirring. Si NPs were the largest NPs investigated (100 nm diam. primary size) and the lower amount of copper adsorbed onto Si NPs compared to smaller TiO<sub>2</sub> NPs used in the present study, can be attributed to the limited surface area available. The study of Si NPs showed that additional analytical methods to chemistry (i.e. *in vivo* investigation of bioavailability) can enhance our understanding about co-contaminant sorption to NPs

contributing to unique information, both concluding that Cu sorption onto Si NPs was very low. Specifically, the sensitive chemical analysis identified the small amount of copper adsorbed on Si NPs, however the presence of Si NPs did not change Cu toxicity of algae or zebrafish indicating that is bioavailability as a detection method of lower sensitivity but occurs directly in the aquatic environment.

Sorption of copper on the surface of TiO<sub>2</sub> NPs and bulk TiO<sub>2</sub> was indicated by the reduced bioavailability of copper in both algae and zebrafish larvae in the presence of the TiO<sub>2</sub> NPs.

The smaller diameter of TiO<sub>2</sub> NPs indicates greater surface area and reactive sites for sorption by the NPs (Zhang et al., 1998); however, agglomeration of TiO<sub>2</sub> NPs, that can occur at higher concentration ranges (i.e. 4-16 mg/L of TiO<sub>2</sub> NPs) as demonstrated in Figure 1B or higher pH levels (i.e. the fresh water media used in the present study), can strongly change the reactivity of the particles by decreasing the capacity of aqueous ion adsorption (Gilbert et al., 2009; Suttiponparnit et al., 2011). The importance of NP surface area on copper sorption was supported by the gene expression analysis. Boran et al. (Boran et al., 2016) indicated the importance of surface area in sorption processes when Hg<sup>2+</sup> became less bioavailable to zebrafish larvae in the presence of TiO<sub>2</sub> NM105 compared to TiO<sub>2</sub> bulk. *Mt2* induction in the present study can be indicated as a fast and direct measurement of copper bioavailability and effectively demonstrated copper sorption on the smallest TiO<sub>2</sub> NPs.

Copper sorption to TiO<sub>2</sub> NPs reduced concentration of bioavailable copper to a level low enough to significantly decrease copper-induced growth inhibition, mortality and *mt2* expression in *C. vulgaris* and zebrafish larvae, respectively. Several studies have supported sorption of aqueous metals such as cadmium (Hartmann et al., 2010; Yang et al., 2012) and chromium (IV) (Dalai et al., 2014) on TiO<sub>2</sub> NPs. Fan et al (Fan et al., 2012) reported an increased activity of superoxide dismutase after sorption of Cu onto TiO<sub>2</sub> NPs and a positive correlation between the enzyme activity and mortality in *D. magna* in the presence of 2 mg/L

TiO<sub>2</sub> NPs after Cu sorption onto TiO<sub>2</sub> NPs. However, reduced toxicity of copper, presumably as a consequence of aqueous-phase copper sorption to TiO<sub>2</sub> NM105, has been reported using the same organism (Rosenfeldt et al., 2014; Rosenfeldt et al., 2015). Specifically, Rosenfeldt and colleagues (Rosenfeldt et al., 2014) observed that TiO<sub>2</sub> NPs agglomerates were actively ingested by *D. magna* but no release of adsorbed copper in the gastrointestinal tract was observed. In the present study, although *C. vulgaris* came in direct contact with NP agglomerates with sorbed copper (constant agitation), the free-swimming zebrafish larvae (static test) were less likely to have physical contact or actively ingest the settled TiO<sub>2</sub> agglomerates with adsorbed Cu at the early developmental stage of 96 hpf. The inability of 96-hpf zebrafish larvae to actively ingest micro-scale NP agglomerates (visible with naked eye at the bottom of glass beakers), may have lessened the likelihood of NPs to act as a delivery vehicle for copper to the organism and therefore, led to decreased bioavailability of co-contaminant. Although assessing bioavailability of an adsorbed co-contaminant does not inform on specific co-contaminant and NP surface interaction, it does inform directly on potential implications by the presence of NPs in the aquatic environment.

The present study provided unique insight in the sorption of copper to a wide range of different NPs and how NP sorption properties can affect the aquatic environment. Analytical chemistry, algal growth inhibition, zebrafish larvae mortality and zebrafish gene expression identified a positive relationship between copper sorption and surface area of NPs, using a wide range of copper concentrations. The close similarity of the slopes of the sorption (%) and surface area relationship among methodologies (Graphical Abstract) suggest increasing sorption with surface area (calculated after the reported manufactured particle diameter), that can establish the surface area as the strongest factor affecting sorption in the present study. Accepting a linear relationship of sorption with surface area, the highest intercept potentially indicates highest copper sorption capacity of the NPs that is identified by each sorption

investigation tool. Therefore, the data provided using different Cu and NP concentration ranges in two model organisms with different exposure approaches, the 96-h zebrafish larvae LC<sub>50</sub> and 72-h *C. vulgaris* EC<sub>50</sub>, can inform effectively on sorption capacity of the NPs compared to sensitive gene expression and ICP-MS analyses after a 24-h exposure. Without having investigated the underlying mechanisms of metal ion sorption on NPs, taken together the findings of the present study indicate that sorption of copper can differ between different materials with the same surface charge (i.e. Si, perovskite and TiO<sub>2</sub> NPs) or between different physicochemical properties of the same material (e.g. TiO<sub>2</sub> NPs of different size or shape). This study also demonstrates the importance of bioavailability as a unique evaluation tool of the direct effect of sorption of copper on NPs contributing to an environmentally relevant assessment of the impact of NPs in the aquatic environment.

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## FIGURE CAPTIONS

Graphical Abstract: Copper sorption (%) as indicated by four independent analyses, conducted in the present study, show a positive correlation with the surface area (calculated by the reported manufactured particle diameter) of the NPs used in the present study ( $\text{cm}^2$ ). The differences in the 72-h  $\text{EC}_{50}$  ( $\Delta$  72-h  $\text{EC}_{50}$ ) in the presence of NP of different surface area generated by *C. vulgaris* tests, the  $\Delta$  96-h  $\text{LC}_{50}$  of the zebrafish larvae tests, the differences in *mt2* gene expression and the analytical chemistry (ICP-MS) generated curves with  $R^2$ : 0.90, 0.88, 0.81 and 0.99, respectively.

Figure 1. A) Total Cu concentration (mg/L) measured by ICP-MS in the presence of NM105 (diamonds), Si NPs (circles) and  $\text{TiO}_2$  4-8 nm (triangles). Each point in the graph represents a single measurement. B) The computed mass of total copper missing from the water column [Figure 1A, measured by ICP-MS in the presence of  $\text{TiO}_2$  NPs (4-8 nm)] is assumed to have been adsorbed onto the NPs and is presented by the black line and full black dots. The dotted line represents amount of total copper that would be adsorbed on the surface area of the  $\text{TiO}_2$  (4-8) NPs if a proportional increase in Cu adsorption was observed with increasing concentration of NPs and consequently surface area for Cu adsorption. The ICP data show that Cu adsorption is reaching a plateau that can be attributed to agglomeration of the NPs that in turn would decrease the surface area capacity. Surface area was calculated by the reported manufactured diameter of sphere-shaped Si NPs and NM105 ( $4\pi r^2$ ) and the reported manufactured dimensions of rod-shaped  $\text{TiO}_2$  4-8 nm NPs ( $2r\pi h + 2\pi r^2$ ).

Figure 2: The growth inhibition (%) of the *C. vulgaris* over the range of Cu concentrations (solid lines) in association with 2 mg/L (dashed line) A)  $\text{TiO}_2$  bulk, B)  $\text{TiO}_2$  NM105, C)  $\text{TiO}_2$  18 nm NP and D)  $\text{TiO}_2$  4-8 nm NPs for 72 h, compared to the growth inhibition caused by Cu alone. There was a significant difference in the concentration response curves between Cu

and Cu with TiO<sub>2</sub> NP (B, C and D) indicating that the presence of TiO<sub>2</sub> NPs reduced the bioavailability of Cu (logistic regression,  $p < 0.001$ ). No differences were observed in the presence of TiO<sub>2</sub> bulk.

Figure 3: The mortality (%) of the zebrafish larvae over the range of Cu concentrations (solid line) in association with 2 mg/L (dashed line) A) TiO<sub>2</sub> bulk, B) TiO<sub>2</sub> NM105, C) TiO<sub>2</sub> 18 nm NP and D) TiO<sub>2</sub> 4-8 nm for 96 h, compared to the larvae mortality caused by Cu alone. There was a significant difference in the concentration response curves between Cu and Cu with TiO<sub>2</sub> NPs and bulk (A, B, C and D) indicating that the presence of TiO<sub>2</sub> NPs reduced mortality caused by Cu (logistic regression,  $p < 0.001$ ).

Figure 4: A) Copper concentration-response curve (solid line) and copper with 4 mg/L of NM105 concentration-response curve after 96-h exposure to zebrafish larvae. The difference between LC<sub>50</sub> value of copper/NM105 mixture and copper alone is presented as  $\Delta$  LC<sub>50</sub>. The concentration response curves from which the LC<sub>50</sub> values were calculated can be found in figure S5. B) Increase in the  $\Delta$  LC<sub>50</sub> with increased NM105 concentration. The NM105 added in the copper dilution series ranged between 1 and 4 mg/L. The difference between 96-h LC<sub>50</sub> of copper alone and 96-h LC<sub>50</sub> of copper with NM105 is increasing significantly with concentration of NM105 added in the dilution series.

Figure 5: Induction of *mt2* gene in zebrafish larvae after a 24-h exposure to copper (Cu), copper and 2 mg/L TiO<sub>2</sub> 4-8 nm (CuTiO<sub>2</sub>4-8), copper and 2 mg/L TiO<sub>2</sub> 18 nm diam. (CuTiO<sub>2</sub>18), or copper and 2 mg/L Si NM (CuSi). No significant difference was observed between Cu and CuTiO<sub>2</sub> 18nm or CuSi ( $p > 0.05$ ) but Cu *mt2* expression was found significantly different from CuTiO<sub>2</sub> 4-8 nm, GLM,  $p < 0.001$ . Data represent MEAN $\pm$ SE,  $n=3$ .

Figure 1

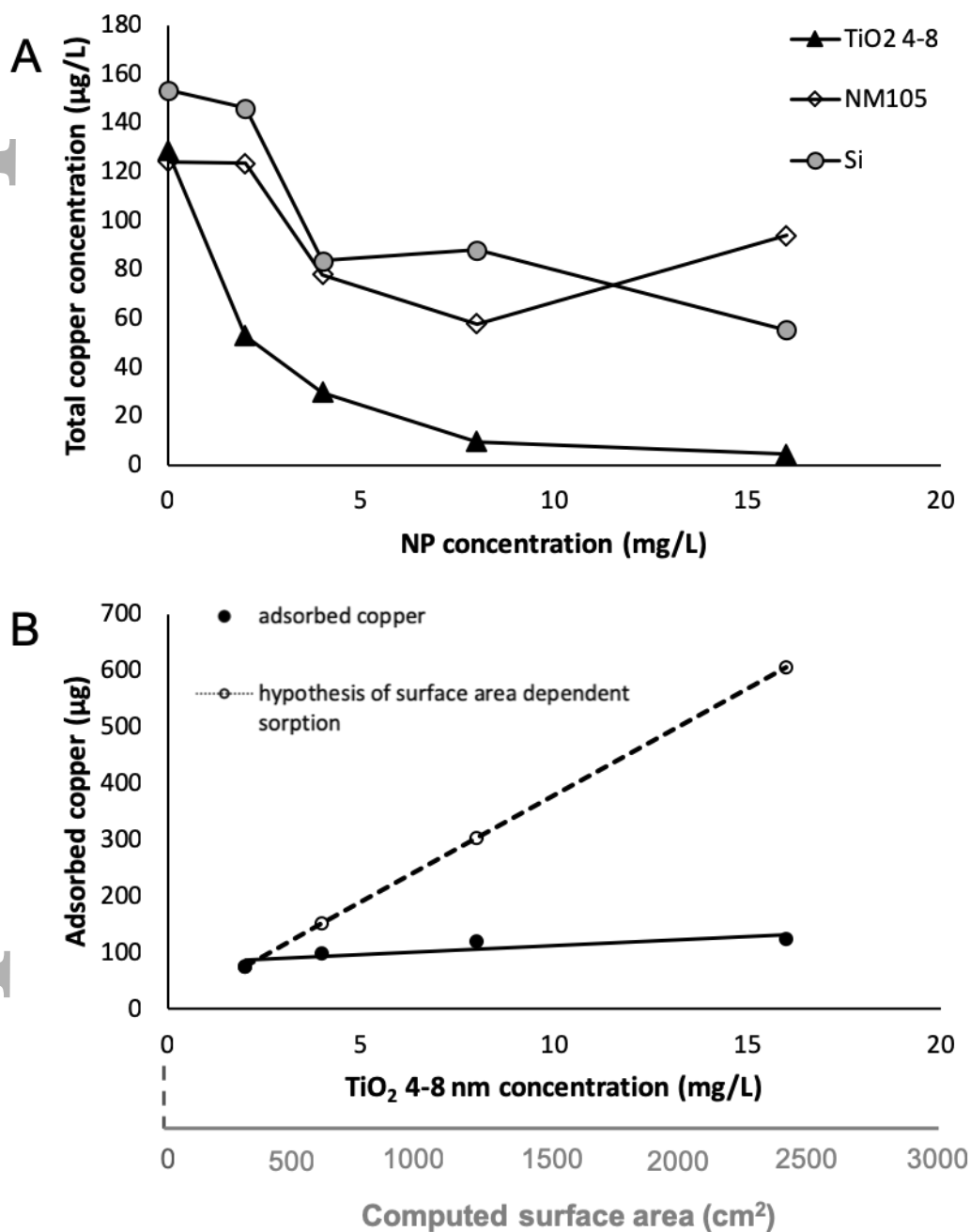


Figure 2

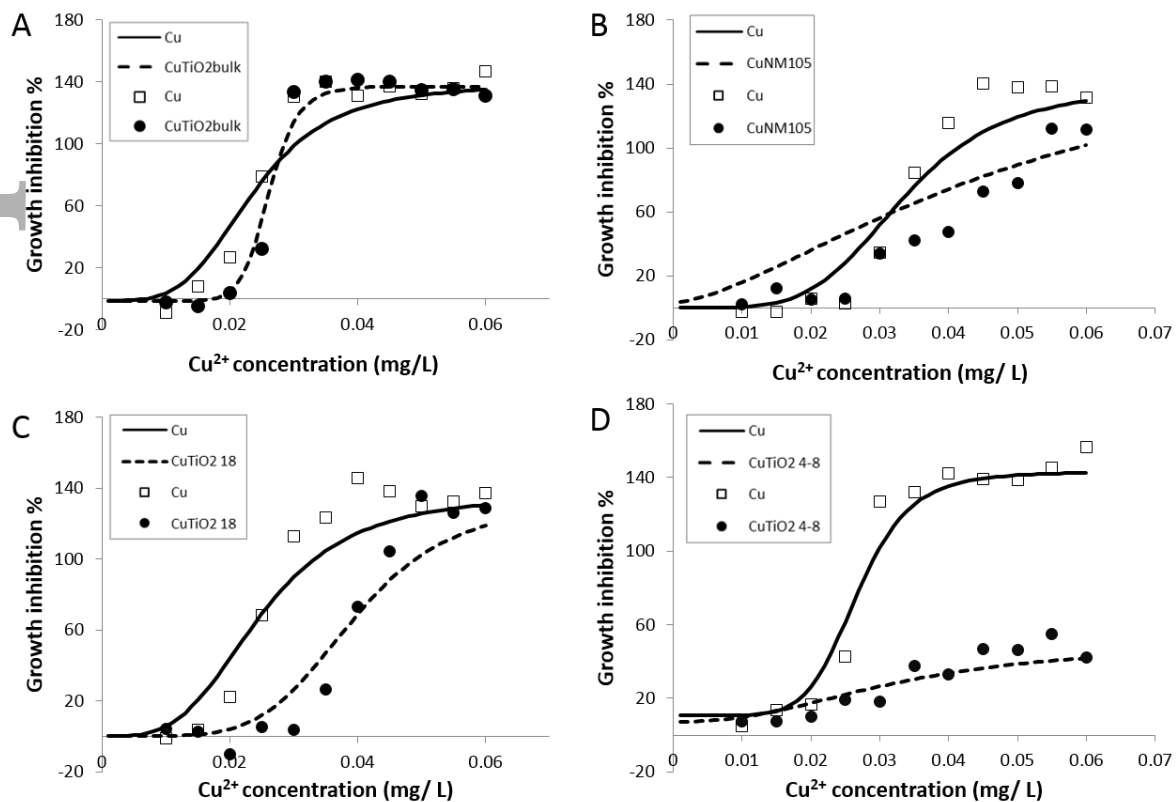


Figure 3

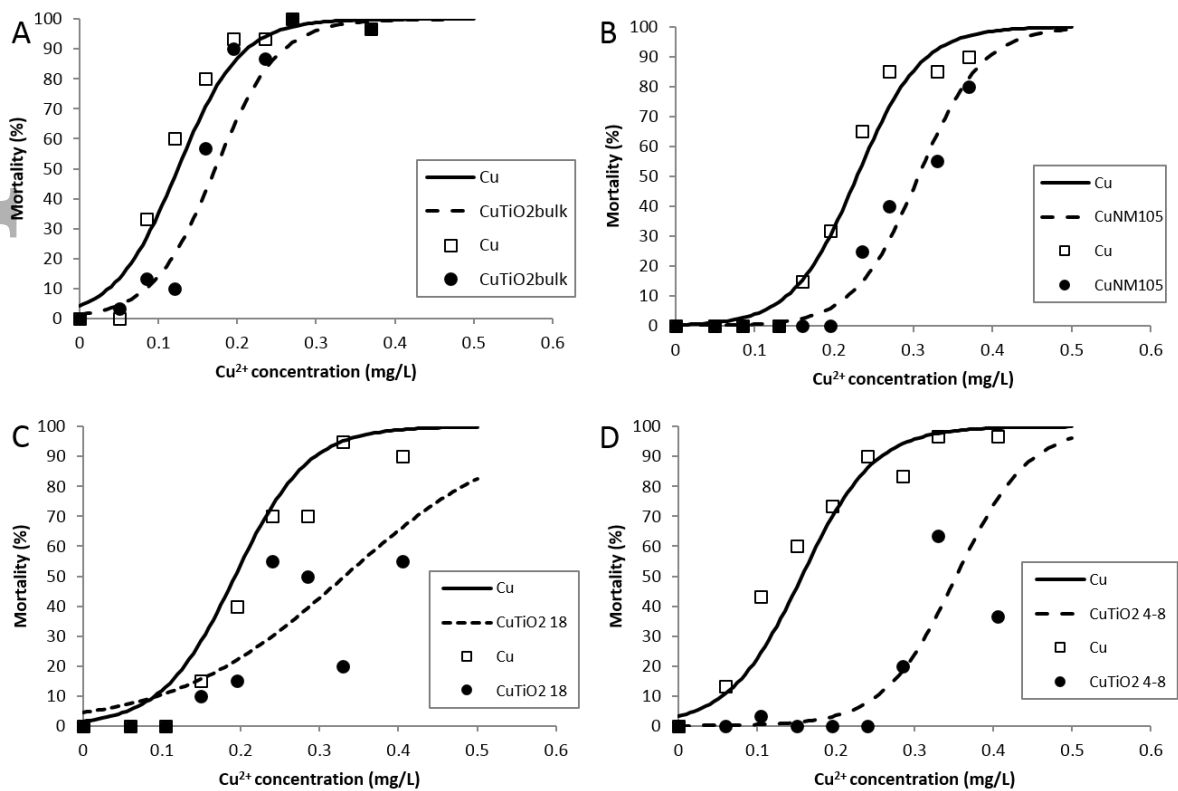


Figure 4

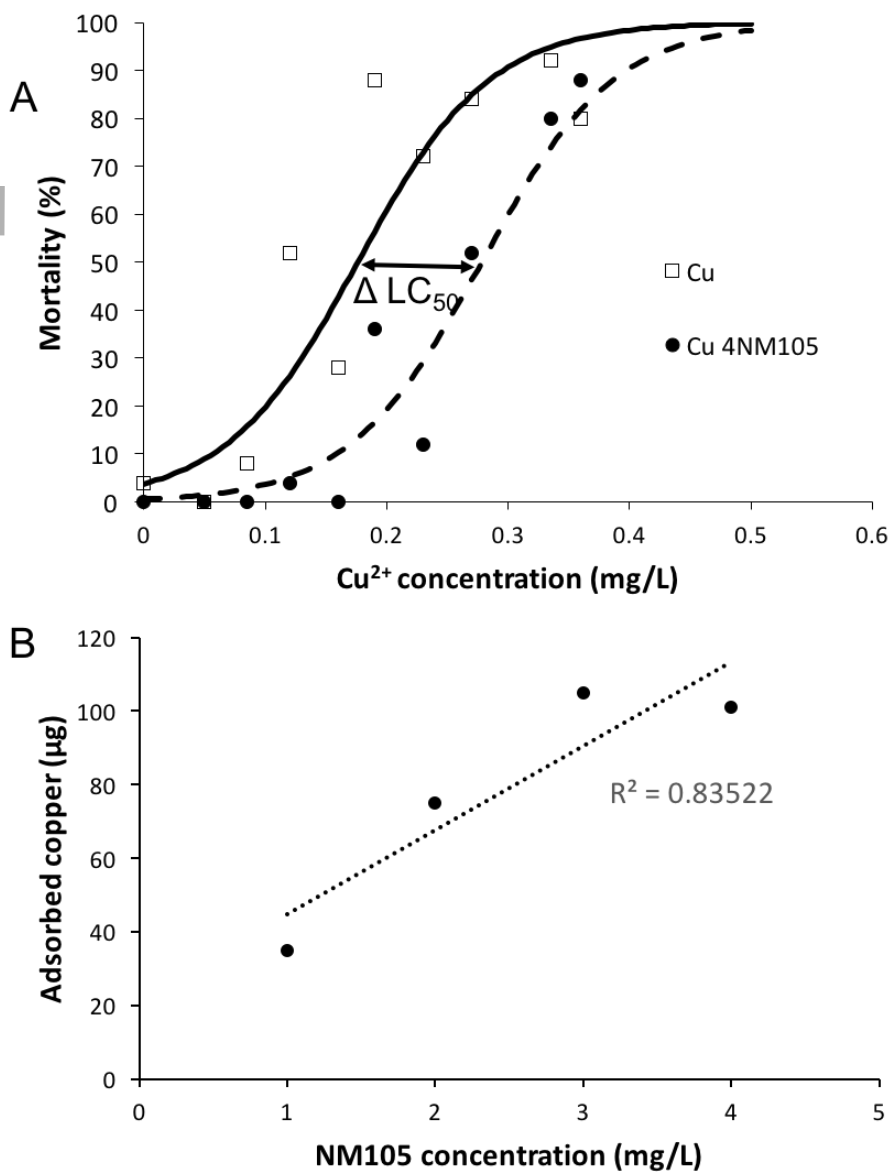




Figure 5

