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# Aqueous $\text{Hg}^{2+}$ associates with $\text{TiO}_2$ nanoparticles according to particle size, changes particle agglomeration, and becomes less bioavailable to zebrafish

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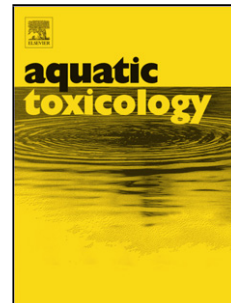
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Author: Halis Boran David Boyle Ilhan Altinok Danae Patsiou Theodore B. Henry



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# Aqueous Hg<sup>2+</sup> associates with TiO<sub>2</sub> nanoparticles according to particle size, changes particle agglomeration, and becomes less bioavailable to zebrafish

*Halis Boran,<sup>1,2,3</sup> David Boyle,<sup>3\*</sup> Ilhan Altinok,<sup>2</sup> Danae Patsiou,<sup>4</sup> Theodore B. Henry,<sup>3,4,5</sup>*

<sup>1</sup>Recep Tayyip Erdoğan University, Faculty of Fisheries, 53100 Rize, Turkey

<sup>2</sup>Karadeniz Technical University, Department of Fisheries Technology Engineering, Faculty of Marine Science, 61530 Sürmene, Trabzon, Turkey

<sup>3</sup>School of Biological Sciences, Plymouth University, Plymouth, UK, PL4 8AA

<sup>4</sup>School of Life Sciences, Heriot-Watt University, Edinburgh, Lothian EH14 4AS, UK

<sup>5</sup>Department of Forestry Wildlife and Fisheries, and Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN 37996, USA

\*Corresponding author address: School of Biological Sciences, Plymouth University, Plymouth PL4 8AA, United Kingdom; phone: +44 (0)1752 584604; FAX: +44 (0)1752 585858; email: david.boyle@plymouth.ac.uk

Highlights - Boran et al. 2015

- TiO<sub>2</sub>-NPs reduced bioavailability of aqueous-phase Hg<sup>2+</sup> to zebrafish larvae
- Reduction in bioavailability of Hg<sup>2+</sup> is related to surface area of TiO<sub>2</sub>-NPs
- Sorption of Hg<sup>2+</sup> increased TiO<sub>2</sub>-NP agglomerate size and deposition to sediment

## Abstract

Engineered nanoparticles (NPs) have unique physicochemistry and potential to interact with other substances in the aqueous phase. Here, gene [*metallothionein 2 (mt2)*] expression changes in larval zebrafish were used to evaluate the association between aqueous Hg<sup>2+</sup> and TiO<sub>2</sub> (NPs and bulk particle size control) to investigate the relationship between changes in Hg<sup>2+</sup> behavior and TiO<sub>2</sub> size. During 24 h exposures, TiO<sub>2</sub> agglomerates increased in size and in the presence of 25 µg Hg<sup>2+</sup> /L, greater increases in size were observed. The concentration of Hg<sup>2+</sup> in suspension also decreased in the presence of TiO<sub>2</sub>-NPs. Mercury increased expression of *mt2* in larval zebrafish, but this response was lessened when zebrafish were exposed to Hg<sup>2+</sup> in the presence of TiO<sub>2</sub>-NPs, and which suggests that TiO<sub>2</sub>-NPs alter the bioavailability of Hg<sup>2+</sup> to zebrafish larvae. This ameliorative effect of TiO<sub>2</sub> was also likely due to surface binding of Hg<sup>2+</sup> because a greater decrease in *mt2* expression was observed in the presence of 1mg/L TiO<sub>2</sub>-NPs than 1 mg/L TiO<sub>2</sub>-bulk. In conclusion, the results show that Hg<sup>2+</sup> will associate with TiO<sub>2</sub>-NPs, TiO<sub>2</sub>-NPs that have associated Hg<sup>2+</sup> will settle out of the aqueous phase more rapidly, and agglomerates will deliver associated Hg<sup>2+</sup> to sediment surfaces.

**Keywords:** Titanium dioxide nanoparticles; zebrafish; metallothionein; mercury; sorption

## 1. Introduction

Among the concerns for negative environmental effects of engineered nanomaterials is the potential that toxic substances can associate with nanoparticles (NPs) and that these associations will influence the environmental fate and toxicity of both NPs and associated substances (e.g. Baun et al. 2008; Henry et al. 2013). High surface to volume ratios for NPs are a consequence of their nanoscale [NPs are defined to have all three external dimensions between 1 and 100 nm (ISO 2008, 2010)], and the large surface areas present extensive opportunities for substances to become associated. Of importance is the potential that toxic substances become associated with nano-sized particles, and this association alters the environmental fate and transport of the substance leading to changes in substance bioavailability including exposure of organisms not otherwise likely to be exposed to the associated substances (Zhang et al. 2007). Toxic substances may become associated with anthropogenic particles either during particle or product manufacturing, during use of the product (e.g., if particles are used in cleaning etc.), after particles enter wastewater streams and treatment processes, or after particles are released and interactions occur with toxicants already present in the environment (Kim et al. 2009).

The physicochemical properties of NPs and characteristics of their surfaces influence their behaviour in the aqueous phase and their associations with substances present in the water (Klaine et al. 2008). For example, substances present in the aqueous phase may associate with NPs because of hydrophobic interactions in which the substance sorbs to the NP to acquire a lower energy state (e.g. reduced hydrogen bond formation), and examples include sorption to agglomerates of  $(C_{60})_n$  (frequently termed  $nC_{60}$ ) by organic substances such as phenanthrene (Baun et al. 2008) or  $17\alpha$ -ethinylestradiol (Park et al. 2011). Metallic NPs (e.g.  $TiO_2$ ) can also surface-bind pollutants, including metal ions, and this may alter chemical speciation in the environment and bioavailability to organisms. For example, in the

presence of sunlight, TiO<sub>2</sub>-NPs have been shown to oxidise surface-bound As(III) to As(V) and thereby increase bioavailability of total As to carp [*Cyprinus carpio* (Sun et al. 2009)].

Inorganic mercury (Hg<sup>2+</sup>) is an environmental neurotoxicant of concern due to its propensity to bioaccumulate in aquatic organisms, biotransform to methylmercury and biomagnify through food chains (e.g. see review by Kidd and Batchelar, 2012). In fish exposed via water (the principal route of Hg<sup>2+</sup> exposure), Hg<sup>2+</sup> has been shown to cause oxidative stress, including lipid peroxidation (Berntssen et al. 2003), which may lead to necrosis in tissues, including brain (Wobeser, 1975). Estimates of global emissions of Hg<sup>2+</sup> amount to approximately 2300 tonnes per annum and originate from both anthropogenic (principally coal combustion) and geochemical processes (Pacyna et al. 2010). TiO<sub>2</sub>-NPs are also emerging surface water contaminants: recent modelled estimates of production (10,200 tonnes in EU) and mass flows predict transfer of TiO<sub>2</sub>-NPs from production and consumption to waste water (Sun et al. 2014). The much greater estimate of annual production of TiO<sub>2</sub>-pigment in the EU (1,510,000 tonnes, Sun et al. 2014), which is broadly equivalent to the TiO<sub>2</sub> bulk used herein, may also contain a nano-sized fraction (Weir et al. 2012). Understanding how Hg<sup>2+</sup> toxicity and bioavailability in fish are affected by the presence of other aqueous contaminants e.g. TiO<sub>2</sub>-NPs in the water column is therefore an important aspect of risk assessment for both contaminants.

In light of the above information, the aims of the present study were: (1) to investigate particle size, concentration, and aggregation characteristics of TiO<sub>2</sub>-bulk (particle size control) and -NPs in the presence or absence of Hg<sup>2+</sup>, and (2) to evaluate the effects of the association of TiO<sub>2</sub>-NPs and TiO<sub>2</sub>-bulk with Hg<sup>2+</sup>, on the response of zebrafish (*Danio rerio*) larvae to Hg<sup>2+</sup>, by assessing expression of *metallothionein 2* (*mt2*). Metallothioneins are low-molecular-weight proteins rich in cysteine residues with primary functions as intracellular antioxidants and chaperones for Zn<sup>2+</sup> (Chiaverini and De Ley, 2008; Colvin et al. 2008).

Metallothioneins also bind other metal ions (e.g.  $\text{Hg}^{2+}$  and  $\text{Cd}^{2+}$ ) with higher affinity than  $\text{Zn}^{2+}$ , and induction of mt synthesis by displaced  $\text{Zn}^{2+}$  or directly by other metal ions themselves (Kling and Olsson, 1995), is an often used biomarker of metal exposure and accumulation. For example, Monteiro et al. (2010) report increased mt in liver, gill and heart of juvenile matrinxã (*Brycon amazonicus*, an Amazonian fish) exposed to  $\text{HgCl}_2$ .

## 2. Materials and methods

### 2.1 Experimental fish

Broodstock zebrafish (age 4-5 months) were maintained in conditioned dechlorinated Plymouth City tapwater in re-circulating aquaria systems within the Zebrafish Research Facility at Plymouth University and under routine ethically approved animal welfare protocols. Water quality parameters were measured daily (mean  $\pm$  S.D.) for temperature ( $26 \pm 1^\circ\text{C}$ ), pH  $6.7 \pm 0.3$ , and dissolved oxygen ( $92 \pm 3\%$ ), and ammonia, nitrite and nitrate were analysed weekly ( $< 0.02$ ,  $< 0.1$  and  $< 20$  mg/L, respectively) to ensure water quality complied with animal welfare protocols. Concentrations of major cations in water can be found in Sovová et al. (2014). The photoperiod was 12:12 h (light: dark). Fish were fed three times daily with live brine shrimp nauplii (*Artemia* spp.) and dry fish flake mix (equal proportions of ZM Systems flake, dried brine shrimp, spirulina, and TetraMin® stable flake).

To obtain embryos, stock fish were bulk spawned over nets. Embryos were collected within 2 h of fertilization, debris and unfertilized eggs were removed and embryos were reared in 50 mL Petri dishes with daily water changes until 72 hpf when exposures commenced with hatched larvae. Larvae with malformations or of small size were not used in exposures.

### 2.2 Preparation of $\text{TiO}_2$ -NP and $\text{TiO}_2$ -bulk suspensions

The  $\text{TiO}_2$ -NP and  $\text{TiO}_2$ -bulk materials used herein were from the same batches previously characterised and used by our laboratory (e.g. Boyle et al. 2013a, 2013b, 2015) and

for consistency the preparation of stock suspensions in the present study followed the same protocols. Briefly, dry, powdered TiO<sub>2</sub>-NPs (“Aeroxide” P25 TiO<sub>2</sub>, Degussa AG), were supplied by Lawrence Industries, Tamworth, UK. According to the manufacturer, TiO<sub>2</sub>-NPs had a purity > 99 % TiO<sub>2</sub>, comprised 75% anatase and 25% rutile TiO<sub>2</sub> and had a specific surface area of  $50 \pm 15 \text{ m}^2/\text{g}$ . Bulk TiO<sub>2</sub> powder was obtained from ACROS Organics (New Jersey, USA) and had a TiO<sub>2</sub> purity of 98.5 – 100.5%. Primary particle size analyses performed by Boyle et al. (2013a) with electron microscopy indicated that sizes of TiO<sub>2</sub>-NPs and -bulk were (mean  $\pm$  S.D.,  $n = 100$  particles):  $24 \pm 10$  and  $134 \pm 42$  nm, respectively. Dispersed stock suspensions of TiO<sub>2</sub> at 20 mg/mL in ultrapure Milli-Q (Millipore) water were prepared in acid-washed bottles and were generated without solvents with sonication (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 1 h. Mercuric chloride (HgCl<sub>2</sub>) was obtained from BDH Chemicals Ltd (> 97%, Poole, UK) and prepared as a stock solution in ultrapure Milli-Q water (Millipore) in an acid-washed bottle.

### *2.3 Zebrafish larvae exposures and characterisation of exposure media*

Exposures [and characterizations of the interactions between TiO<sub>2</sub> (bulk and NPs) and Hg<sup>2+</sup>] were performed in conditioned Plymouth City tap water (with parameters as described above) in 2-L glass test chambers as previously described by Boyle et al. (2015), with continuous aeration and containing a semi-isolated mesh bottomed container suspended in the upper third to house the larvae. This design allowed for the homogenous distribution of the test materials throughout the chamber at  $t = 0$  h but isolated the zebrafish larvae away from the bottom of the beaker. Previously, we demonstrated that this exposure chamber design maintained approximately 80% of the initial time 0 concentrations of NPs in suspension at 96 h compared to < 5% of material remaining in suspension in static beakers (Boyle et al. 2015).

Two separate exposures with zebrafish larvae were performed: firstly, in combination with variable concentrations of TiO<sub>2</sub>-NPs (0.01-10 mg/L) to determine the concentration-



dependent relationship between TiO<sub>2</sub>-NPs and *mt2* induction ( $n = 1$  chamber per concentration); and secondly, in combination with 1 mg/L TiO<sub>2</sub>-NPs and TiO<sub>2</sub>-bulk (a particle size control), and with greater replication ( $n = 3$  chambers per concentration), to test the hypothesis that particle size also exerts an influence on Hg<sup>2+</sup> bioavailability. The usefulness of *mt* as a bioavailability indicator of metal exposure has been questioned (Mieiro et al. 2011) and its use requires validation within a test system. Previously, we have demonstrated the concentration dependent induction of *mt2* expression in zebrafish larvae exposed to sublethal concentrations of  $\leq 25$   $\mu\text{g/L}$  Hg<sup>2+</sup> for 24 h from 72 h post fertilization [hpf (Henry et al. 2013)]. This approach and concentration of Hg<sup>2+</sup> (25  $\mu\text{g/L}$ ) were similarly used in this study. After larvae ( $n = 30$  per chamber) were gently placed into chambers, both TiO<sub>2</sub> and Hg<sup>2+</sup> were immediately dosed into beakers from separate stocks and the exposures commenced for 24 h.

Quantification of Hg in exposure media at 0, 2, 4, 8 and 24 h ( $n = 3$  replicate chambers) was performed using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Varian 725-ES) and calculated from matrix-matched acidified element (Hg<sup>2+</sup>) standards. Concentrations of Hg<sup>2+</sup> in Plymouth tapwater were below the instrument detection limits of 1  $\mu\text{g/L}$ . Attempts to quantify the concentrations of Ti in TiO<sub>2</sub> stock suspensions and exposure media gave poor and variable recoveries and the values given in figures and discussion are based on the nominal concentrations of the initial ones.

Characterizations of TiO<sub>2</sub> in the aqueous phase in exposure water (Plymouth tapwater) were conducted by nanoparticle tracking analysis (NTA) with a NanoSight LM10 (NanoSight Ltd, Amesbury, UK) particle analyser and standardized settings between treatments/ replicates. The 2-L suspensions were prepared as described for exposures above i.e. 1 mg/L TiO<sub>2</sub>-NPs or TiO<sub>2</sub>-bulk particles and with/ without 25  $\mu\text{g/L}$  Hg<sup>2+</sup>. Samples were collected from within the semi-isolated chamber that would house the larvae and analyzed immediately. Three independent samples were collected from both the TiO<sub>2</sub> (NPs or bulk particles) and

TiO<sub>2</sub> (NPs or bulk particles) + Hg<sup>2+</sup> conditions at 0 and 24 h, and mean particle size distribution plots were computed.

#### 2.4 Gene expression analyses

Total RNA was extracted from pooled samples of  $n = 30$  larvae snap frozen in liquid N<sub>2</sub> at the end of exposures and stored at -80°C until required, using the RNeasy MiniKit for animal tissue (Qiagen, West Crawley, UK) and following manufacturer's protocol. Briefly, larvae were homogenised in RLT buffer with sonication (3-5 s) followed by additional tissue disruption in QiaShredder columns. All RNA samples were DNase treated (on-column, Ambion) during extraction. After quantification of total RNA and assessment of impurities with NanoDrop (ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA), 800 ng were used to synthesize cDNA following the manufacturer's protocol for ImProm-II™ Reverse Transcription System (Promega, Southampton, UK) in a reaction mixture containing hexanucleotide primers.

qPCR was performed using cDNA diluted 1 in 10 with nuclease free H<sub>2</sub>O, with 375 nmol gene specific primers (see below) and SYBR Green JumpStart *Taq* ReadyMix in a final reaction volume of 20 µL and using a light cycling PCR instrument (StepOne Real-Time PCR System, Applied Biosystems). Primers specific for zebrafish *mt2* (NCBI Reference Sequence: NM\_001131053.2) and the zebrafish housekeeping gene *β-actin 1* (NCBI Reference Sequence: NM\_131031.1) were obtained from Eurofins UK (Wolverhampton, UK) and were as follows (5'-3'): CTGCGAATGTGCCAAGACTGGAAC *mt2* forward; GCGATGCAAACGCAGACGT *mt2* reverse; ACACAGCCATGGATGAGGAAATCG *β-actin 1* forward; TCACTCCCTGATGTCTGGGTCGT *β-actin 1* reverse). The cycling conditions for qPCR were as follows: 94°C for denaturing, primer-specific annealing 55-60°C, and extension at 72°C. All samples were run in triplicate and the specificity of primers

assessed via in-instrument melt curve analysis and further with 1% gel electrophoresis of qPCR products. The efficiency of qPCR was calculated based on a 4- or 5-point standard curve run on each plate and normalised across runs. Efficiencies between 90-110% were accepted for further analyses, and the comparative quantification method ( $2^{-\Delta\Delta C_t}$ ) was used for calculating fold-changes in expression of *mt2* (after normalisation to  *$\beta$ -actin 1*) compared to controls.

### 2.5 Statistical analysis

Statistical analyses and curve-fitting were performed using GraphPad Prism (GraphPad Software, Inc. v. 6). Statistically significant differences in concentrations of  $Hg^{2+}$  in suspensions and level of *mt2* induction were detected using One-Way ANOVA with Tukey's test *a posteriori*. A probability level of  $< 0.05$  was considered to indicate statistical significance. Data are presented as means  $\pm$  S.D.

## 3. Results and discussion

Environmental contaminants do not exist in isolation and the investigation of multiple toxicants in laboratory toxicity tests is necessary to enhance the ecological relevance of gathered data. Mercury is a potent neurotoxin of ecological concern which has significant bioaccumulation potential in fishes (e.g. Berntssen et al. 2003). In zebrafish larvae exposed to  $25 \mu\text{g/L } Hg^{2+}$ , the mRNA expression of *mt2*, a metal-binding protein, was significantly increased ( $18.8 \pm 2.1$  fold greater) compared to the water only controls (Fig. 1), and this is consistent with previous reports (Henry et al., 2013). Exposure of zebrafish larvae to  $TiO_2$  (bulk and NPs) did not cause a change in expression of *mt2* relative to unexposed control fish in the present study (Fig. 2A). Changes in expression of *mt2* were not reported in a study that investigated global gene expression changes in zebrafish embryos exposed to  $TiO_2$ -NPs (Park and Yeo, 2013), and no changes in expression of *mt2* were found by *in situ* hybridization in

zebrafish larvae exposed to TiO<sub>2</sub>-NPs (Osborne et al. 2013). In zebrafish exposed to 25 µg/L Hg<sup>2+</sup>, the expression of *mt2* decreased with concentration of TiO<sub>2</sub>-NPs with a relation that was consistent with a two-phase exponential decay model ( $r^2 = 0.9999$ ) with the *mt2* response to Hg<sup>2+</sup> in zebrafish decreasing with increasing TiO<sub>2</sub>-NPs. At concentrations greater than 1 mg/L TiO<sub>2</sub>-NPs no further additive decrease in *mt2* expression was observed (but expression was still greater than in water only controls at all concentrations of TiO<sub>2</sub>-NPs tested). Previous studies have also shown *mt2* expression to be induced in larval zebrafish upon exposure to Hg<sup>2+</sup> (Chan et al. 2006) and that this response is concentration-dependent (Henry et al. 2013). The lower expression of *mt2* observed in zebrafish may therefore indicate the altered behavior of Hg<sup>2+</sup> in suspension when in the presence of TiO<sub>2</sub>-NPs and decreased bioavailability to zebrafish.

Sorption of environmental contaminants with NPs in the aqueous phase has been documented and used to explain changes in contaminant bioavailability in organisms, including fishes (Henry et al. 2013; Knauer et al. 2007). The association of metal ions and NPs is likely to be electrostatic in nature such as between the positively charged Hg<sup>2+</sup> ion and the negatively charged TiO<sub>2</sub>-NPs in the present study. Previous studies have indicated that the sorptive potential of nanomaterials is a function of both the agglomeration behaviour and surface area variation of NP dispersions and that this can have an effect on both the reactivity of nanomaterials and their efficiency in contamination treatment (Gilbert et al. 2009; Jassby et al. 2012; Zeng et al. 2009). The effect of particle size (surface area) on Hg<sup>2+</sup> behavior and bioavailability was further explored by co-exposing zebrafish larvae to Hg<sup>2+</sup> in combination with 1 mg/L TiO<sub>2</sub>-NPs and 1 mg/L TiO<sub>2</sub>-bulk (Fig. 2). Expression of *mt2* was significantly lower in larvae co-exposed to NPs compared to bulk and both materials had significantly decreased *mt2* compared to larvae exposed to Hg<sup>2+</sup> alone (Fig. 2A). This is consistent with the hypothesis that particle surface area, perhaps as a determinant of sorptive potential for Hg<sup>2+</sup>,

decreases  $\text{Hg}^{2+}$  mobility in suspension. In part, the decrease in *mt2* expression effect was due to the decreased concentration of  $\text{Hg}^{2+}$  in the water column (Fig. 2B). Compared to 0 h ( $22.1 \pm 0.7 \mu\text{g/L}$ ), after 24 h the concentration of  $\text{Hg}^{2+}$  in the chambers in the  $\text{Hg}^{2+}$  only positive control were unchanged ( $21.8 \pm 1.2 \mu\text{g/L}$ ), but had significantly decreased to  $14.0 \pm 1.6 \mu\text{g/L}$  in chambers in the presence of  $\text{TiO}_2$ -NPs. The chamber design used herein has been previously shown to better maintain homogeneous dispersions of NPs than static exposures in unstirred beakers (Boyle et al. 2015); however, a small amount of settling out was expected and this possibly led to the decrease in the concentration of  $\text{Hg}^{2+}$  measured in the NP +  $\text{Hg}^{2+}$  suspension. Interestingly, a significant decrease in  $\text{Hg}^{2+}$  was not observed in the presence of  $\text{TiO}_2$ -bulk ( $19.4 \pm 0.2 \mu\text{g/L}$  after 24 h) and the significant decrease observed in *mt2* induction (Fig. 2A) was therefore not (only) a function of the sedimentation of  $\text{Hg}^{2+}$  associated with  $\text{TiO}_2$ -bulk (Fig. 2B). At 1 mg/L,  $\text{TiO}_2$ -NPs and  $\text{TiO}_2$ -bulk have been shown to occlude epithelia in fish and cause an increase in mucus production (Boyle et al. 2013a) and this could act as a physical barrier to  $\text{Hg}^{2+}$  uptake. More likely,  $\text{Hg}^{2+}$  sorbed to  $\text{TiO}_2$  is less available for uptake via branchial/ cutaneous ion transport pathways.

Nanoparticle tracking analyses of aqueous  $\text{TiO}_2$ -NPs and  $\text{TiO}_2$ -bulk preparations indicated that  $\text{TiO}_2$  formed agglomerates and settled out of the water column over the 24 h exposure periods (Fig. 3). In general, and across all treatments, the data indicated that  $\text{TiO}_2$  was dosed as numerous small particles (and agglomerates) at 0 h, and 24 h later these agglomerates were fewer in number greater in diameter. In the presence of  $25 \mu\text{g/L}$   $\text{Hg}^{2+}$ ,  $\text{TiO}_2$ -NPs agglomerates were also greater in size (Fig. 3A). The behavior of  $\text{TiO}_2$ -NPs (including P25) in suspensions with divalent metals, especially  $\text{Ca}^{2+}$ , is well characterized; sorption of  $\text{Ca}^{2+}$  to the surface of  $\text{TiO}_2$ -NPs neutralizes negative surface charge which decreases the electrostatic repulsive forces between NPs leading to NP agglomeration

(Domingos et al. 2010; von der Kammer et al. 2010). A similar effect of  $\text{Hg}^{2+}$  could explain larger agglomerates of  $\text{TiO}_2$ -NPs formed in the presence of  $\text{Hg}^{2+}$ . Mercury also had less of an effect on the particle size distributions of  $\text{TiO}_2$ -bulk in suspension after 24 h (Fig. 3B). These observations and data of *mt2* induction and  $\text{Hg}^{2+}$  concentrations in suspension with  $\text{TiO}_2$ -bulk are broadly supportive of the hypothesis that particle surface area determines extent of interactions of  $\text{TiO}_2$  with  $\text{Hg}^{2+}$ .

In conclusion,  $\text{TiO}_2$ -NPs decreased the concentration and affected the behavior and bioavailability of  $\text{Hg}^{2+}$  in the aqueous phase. This effect was related to the greater surface area of NPs. The observation that  $\text{TiO}_2$ -NPs can sorb toxicants (in this case  $\text{Hg}^{2+}$ ) and this association may alter the delivery of toxicants to organisms located in specific environmental compartments (e.g. increased sedimentation) is environmentally relevant and of toxicological importance. While  $\text{TiO}_2$ -NPs may be of low toxicity to organisms (e.g. fishes, Boyle et al. 2013b, 2015), their potential effect on the fate of co-contaminants in the environment requires further consideration in environmental risk assessment.

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### Figure legends

Fig. 1. Increasing the concentrations of TiO<sub>2</sub>-NPs led to a decrease in expression of *mt2* in zebrafish larvae co-exposed to 25 µg/L Hg<sup>2+</sup> between 72 and 96 h post-fertilization. These data fit a two-phase exponential decay model ( $r^2= 0.9999$ ). Data are expressed relative to controls (no TiO<sub>2</sub> or Hg<sup>2+</sup>) following normalization to *β-actin 1*.

Fig. 2. The expression of *mt2* during exposure to Hg<sup>2+</sup> was affected by co-exposure to different sizes of TiO<sub>2</sub> particles (A), and TiO<sub>2</sub> also changed the concentration of Hg<sup>2+</sup> in the aqueous suspensions (B). Zebrafish larvae at 72 h post-fertilization were exposed to 25 µg/L Hg<sup>2+</sup> for 24 h in combination with 1 mg/L TiO<sub>2</sub>-bulk or TiO<sub>2</sub>-NPs. Expression of *mt2* is shown relative to controls (no Hg<sup>2+</sup> or TiO<sub>2</sub>) and following normalization to *β-actin 1*. Concentrations of Hg<sup>2+</sup> in water only controls were < 1 µg/L (instrument limit of detection, data not shown); ● Hg<sup>2+</sup>; ■ Hg<sup>2+</sup> + TiO<sub>2</sub>-bulk; ▲ Hg<sup>2+</sup> + TiO<sub>2</sub>-NPs. Where errors bars cannot be seen they are not greater than the width of the symbol. Data points with different lower case letters are significantly different (One-way ANOVA, Tukey's test *a posteriori*,  $p < 0.05$ ). Data are means ± S.D.,  $n = 3$ .

Fig. 3. Hg<sup>2+</sup> increased agglomerate size of TiO<sub>2</sub>-NPs. The particle size distributions of 1 mg/L TiO<sub>2</sub>-NPs (A) and 1 mg/L TiO<sub>2</sub>-bulk (B) at 0 and 24 h and in the presence of 25 µg/L Hg<sup>2+</sup> were tracked with Nanoparticle Tracking Analysis. Data are representative traces for ease of visualization of trends.

Fig. 1.

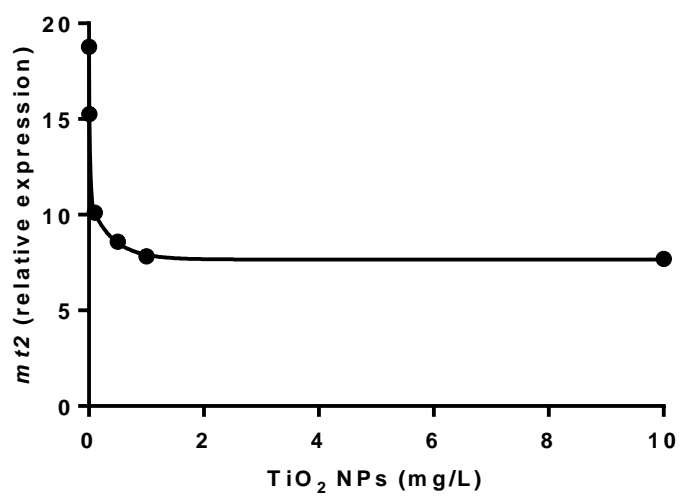


Fig. 2.

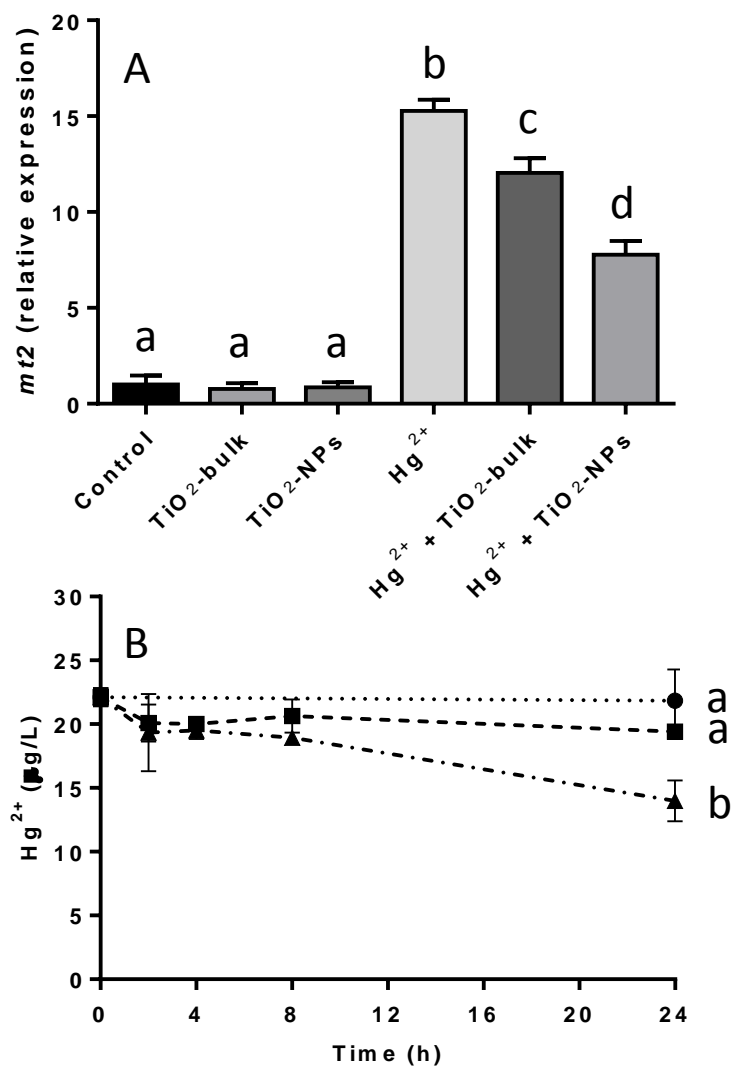


Fig. 3.

