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Research Article

Bioaccumulation, Subacute Toxicity, and Tissue Distribution of Engineered Titanium Dioxide Nanoparticles in Goldfish (*Carassius auratus*)

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The increased use of nanosized materials is likely to result in the release of these particles into the environment. It is, however, unclear if these materials are harmful to aquatic animals. In this study, the sublethal effects of exposure of low and high concentrations of titanium dioxide nanoparticles (TiO_2 NPs) on goldfish (*Carassius auratus*) were investigated. Accumulation of TiO_2 NPs increased from 42.71 to 110.68 ppb in the intestine and from 4.10 to 9.86 ppb in the gills of the goldfish with increasing exposure dose from 10 to 100 mg/L TiO_2 NPs. No significant accumulation in the muscle and brain of the fish was detected. Malondialdehyde as a biomarker of lipid oxidation was detected in the liver of the goldfish. Moreover, TiO_2 NPs exposure inhibited growth of the goldfish. Although there was an increase (8.1%) in the body weights of the goldfish for the control group, in the low and high exposure groups 1.8% increase and 19.7% decrease were measured, respectively. The results of this study contribute to the current understanding of the potential ecotoxicological effects of nanoparticles and highlight the importance of characterization of NPs in understanding their behavior, uptake, and effects in aquatic systems and in fish.

1. Introduction

Nanomaterials are used in a wide range of domestic appliances and household products, in the manufacture of textiles and electronics, as well as medical products and in bioremediation technology. There are also concerns about the environmental risks of nanotechnology which need to be balanced against their undoubted benefits to human society [1, 2]. Handy and Shaw [3] reviewed the risks to human health and identified a number of exposure routes including the discharge of nanoparticles (NPs) to water and agricultural land. The chemistry and physical characteristics of the NPs themselves are key elements in determining their fate and behavior in aquatic systems. The large surface area, crystalline structure, and reactivity of some NPs may facilitate transport of these toxic materials in the environment [4]. Certain conditions such as presence of humic and fulvic acids, pH, and specific cation concentrations may favor the stabilization of NPs in the water column [5]. The aquatic species are also at risk of exposure to the NPs and there is currently little known about their uptake, potential toxic effects, and behavior in aquatic systems.

Titanium dioxide (TiO_2) NPs have been widely used in several industries. Nanoparticulate TiO_2 has been utilized as an ultraviolet radiation absorber in transparent sunscreen formulations [6] and in specialist photocatalytic coatings for glass [7]. The environmental chemistry of TiO_2 NPs has been partly investigated. TiO_2 NPs can be dispersed in freshwater by sonication [8], but the primary particles tend to form aggregates of a few 100 nm dimensions, and the aggregates gradually precipitate from the water column over a few hours. TiO_2 has two major crystal structures (rutile and anastase), and the surface reactivity of the NP is closely defined by the crystal structure [9].

There is an emerging literature on the effects of NPs for fish and aquatic invertebrates. NPs have previously been

shown to accumulate in cells such as macrophages and hepatocytes [10, 11]. Moreover, they are taken up into aquatic organisms such as fish, mollusks, crustaceans, and artemia [12–16]. Fish are excellent sentinels of environmental health as they are sensitive to a wide range of xenobiotic chemicals. Their position in the aquatic food chain means assessment of the populations, and health of fish can give an indication of the health of other lower levels of the food chain. Understanding the effects of NPs on fish is therefore an important aspect when considering the effects of NPs on the aquatic environment as a whole. Potential routes of uptake for NPs in fish include absorption via the gill epithelia, via the intestine epithelia as a result of dietary exposure and drinking, or via the skin [17].

The purpose of this exposure study was to determine subacute toxicity, accumulation, and tissue distribution of engineered TiO_2 NPs in goldfish (*Carassius auratus*). Due to the importance of their size and aggregation behavior [18–20], the NPs were characterized by transmission electron microscopy (TEM), and the size distribution of NPs was measured by dynamic light scattering (DSL). Fish tissues were used as *in vitro* model to determine the possible uptake of TiO_2 NPs into gill, intestine, muscle, and brain. Total Ti accumulation in each tissue was determined by inductively coupled plasma mass spectrometry (ICP-MS). In addition, MDA was determined as a cause of systemic oxidative stress.

2. Materials and Methods

2.1. Test Organism and Experimental Condition. A group of healthy goldfish (*Carassius auratus*) was purchased from a local pet shop. The initial body weight and length of the fish were measured as 4.53 ± 0.06 g and 5.5 ± 0.7 cm, respectively. All fish were maintained in 30 L glass aquarium supplied via a flow-through system with dechlorinated tap water, enriched with oxygen at a temperature of 23 ± 2°C and pH of 6.8. Fish were fed daily with commercially available fish feed flakes (TetraFin Goldfish flakes, Germany) at the amount of 0.5% of mean body weight of the fish. The goldfish were anesthetized using 3-aminobenzoic acid ethylester (MS-222; Aqua Life, Syndel Laboratories Ltd., Vancouver, BC, Canada) at a lethal dose for dissection (excess of 200 mg/L), and a lower dose was used for all handling procedures (150 mg/L).

2.2. Reagents and Chemicals. TiO_2 NPs (TiO_2 10–30 nm NPs, 99.5% pure) were purchased, as uncoated nanomaterials, from Skyspring Nanomaterials Inc., in Houston, TX, USA. TEM image of NPs was spherical with an average particle size (D_{50}) of 10–30 nm and approximate surface area of 50 m^2/g. The morphology of the NPs was rutile with pale yellow color, which is the most widely found polymorph of TiO_2 in nature and in high pressure metamorphic rocks. The TiO_2 NPs were stored at room temperature in the laboratory until the implementation of the experimental studies.

Deionized water produced by Barnstead E-pure system with the resistivity of 18.0 MΩ cm was used to prepare the exposure medium and experimental solutions. Trace metal grade nitric acid (HNO_3, Fisher Scientific) and hydrofluoric acid (HF, 99.99%, Sigma Aldrich) used for dissolution of the goldfish were collected after the exposure to determine the total uptake levels. Stock titanium standard solution (1000 $\mu g\,mL^{-1}$) was purchased from SCP Science (Champlain, NY). Calibration standards for ICP-MS analysis were prepared within a range from 0 to 500 $\mu g\,L^{-1}$ from the stock Ti solution in 5% HNO_3. Carbon coated Cu TEM grids (300 mesh) were purchased from Electron Microscopy Sciences (EMS), Hatfield, PA.

2.3. Characterization of NPs. For preparation of exposure medium, TiO_2 NPs were weighed in polypropylene tubes and dispersed in deionized water. To achieve maximum dispersion, the suspension was homogenized using vortex (Daigger Vortex-Genie 2, Model G560) equipped with a titanium probe. Each suspension was exposed to mixture for about 2 minutes and then immediately transferred into the exposure glass tanks. The characterizations of the TiO_2 NP suspension were performed using TEM and DLS techniques. Size measurements in dried suspension were made by TEM, while DLS provided the size distribution for the hydrated forms of the NPs. In addition, Zeta potential measurements were conducted using the DLS instruments to elucidate the surface charges of the suspensions in the exposure medium.

For TEM measurements, a drop (ca. 8 μL) of solution was allowed to dry on a carbon-coated copper grid overnight (CF300 Cu). The TEM grids were purchased from Electron Microscopy Sciences (EMS). Images were recorded by using JEOL-1011 TEM instrument with 0.2 nm lattice resolution and magnification power up to 106 under the accelerating voltage of 40 to 100 kV. Captured images were analyzed using ImageJ software. For DLS measurement, the protocol followed the standards of the Nanotechnology Characterization Laboratory [21]. A stock solution (10 mg/100 mL) was prepared with deionized water and diluted to a final concentration of 10 μg/mL. Samples were then analyzed with Malvern Zetasizer Model Nano ZS according to manufacturer's protocols. Samples were read in disposable plastic Malvern Cells.

2.4. Experiment Design. NP exposure was conducted to assess acute toxicity and associated behavioral changes on goldfish by exposing the fish to two different doses, 10 and 100 mg/L, of the TiO_2 NPs, using 5 days static tests according to OECD 203 testing guidelines [22]. A control group was also set up without the test compound. Studies were carried out in an aquarium (30 L inner volume). The volume for 10 L level was marked and filled with freshwater followed by addition of the NP suspensions prepared as described above. Sight aeration was provided by a line extending to the bottom of the aquarium. Details of the experimental conditions are summarized in Table 1. Each scheme (control or treatments) was conducted in duplicate with 5 healthy goldfish. Individual length and weight of the fish were measured at the beginning and end of the experiment. The data obtained enabled calculation of the live weight and lengthwise increases and survival rates upon completion of the experiment.

2.5. Instrumental Analysis. At the end of the exposure, fish tissues were sampled for instrumental analysis, about 150 mg of wet tissue was weighed and digested in Teflon vessels

Table 1: Expanded design summary of goldfish (*Carassius auratus*).

Parameter	Control	Group A	Group B
Volume of water in aquarium (L)	10	10	10
TiO_2 NPs concentrations (mg/L)	0	10	100
Duration of exposure (day)	5	5	5
Water temperature (°C)	23 ± 2	23 ± 2	23 ± 2
Oxygen (ppm)	6 ± 1	6 ± 1	6 ± 1
pH value of water (Start–End)	6.30–6.05	6.03–6.15	6.63–6.45
Number of fish within the aquarium	5	5	5
Number of replications	2	2	2

in 2 mL concentrated HNO_3 and 0.5 mL HF for 2 hours using a digestion block (DigiPrep MS, SCP Science) at 160°C according to protocols described elsewhere [23]. At the end, the contents were visually inspected for complete dissolution of TiO_2 NPs (e.g., clear solution without any turbidity) and were diluted to 10 mL with deionized water. The sample solutions were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using a Varian 820MS ICP-MS instrument (Varian, Australia). ICP-MS is a powerful multielement technique for analysis of fish and other aquatic organisms for toxic metals even at subparts per billion levels because of its high sensitivity [24]. Titanium content of the solutions was measured to determine the accumulation pattern of the NPs across the dose of exposure. Total Ti concentration detected was then translated into corresponding TiO_2 NP concentration.

2.6. Oxidative Stress Parameter Analysis. The experiment was designed to allow sublethal physiological effects over the exposure period. The five days exposure time was chosen to enable some physiological or biochemical responses to the test organism. Five fish per treatment were collected from each tank at the end of the experiment for biochemical analysis. The extent of lipid peroxidation in the tissues was determined by measuring the quantity of malondialdehyde (MDA) [25]. Quantification of MDA was done following the methods described by Maness et al. [26], Esterbauer, and Cheeseman [27]. The method is based on the formation of pink MDA-thiobarbituric acid (TBA) adduct which has maximum absorption in acidic solution at 532 nm. Briefly, the liver and muscle tissues were removed separately, immediately frozen in liquid nitrogen, and stored at −20°C until needed. The frozen tissues were rinsed in 9-fold chilled 100 mmol/L, pH 7.8 sodium phosphate buffer solution, and homogenized by a hand-driven glass homogenizer. Approximately 150 mg muscle tissue and 10 μL BHT reagent were immediately transferred into 500 mL cold water in tube and then the sample was homogenized using sonicator (Sonic Dismembrator Model 100, Fisher Scientific). The samples were sonicated on ice by ultrasounds for 2 min at 80% power. All samples and standards were incubated at 90°C for one hour, then centrifuged at 12000 rpm for 15 minutes to separate the suspending tissue. The absorbance of the supernatant (reaction mixture) was measured at 532 nm with HP 8452A model diode array spectrophotometer. The concentration of the MDA formed was calculated based on a standard curve for the MDA (Sigma Chemical Co.) complex with TBA. The extent of lipid peroxidation was expressed in nanomoles (or micromoles) of MDA.

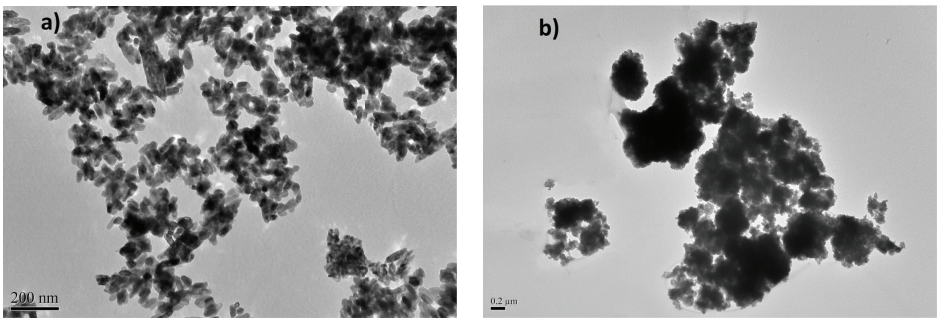
2.7. Statistical Analysis. All experiments were repeated twice independently, and data were recorded as the mean with standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons was used to detect significant differences among groups. Student's t-test was used for paired comparisons of two groups. In all data analyses, a P value <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Characterization of NPs by TEM. TiO_2 NPs are highly hydrophobic; therefore they aggregate substantially in aqueous solutions [28–30]. The stability and aggregation behaviors of NPs within aquatic media are determined by both the physicochemical properties of the liquid and the charge on the surface of the NPs. The degree of aggregation of NPs has been shown in some cases to affect toxicity *in vitro,* and aggregation of the NPs when suspended in water is a known issue for TiO_2 NPs [28, 31]. In this study, the water visibility decreased substantially with increasing concentration of TiO_2 NPs, and at 100 g/L level, the solution was cloudy. Similar aggregation phenomenon has been reported in many NP studies including TiO_2 where aggregates of NPs can sink out of the solution very quickly [32]. The TEM images collected from the dried suspensions of stock solution and exposure medium are illustrated in Figure 1. The TiO_2 NPs aggregated significantly in water yielding large aggregates ranging from around 100 to as high as 1.0 μm in size. Although aeration assisted in maintaining the homogeneity of the suspensions, aggregation could not be avoided at any concentration of TiO_2 NPs.

3.2. Size Distribution and Surface Charge Measurement of NPs. Metal oxide particles tend to aggregate to various extents in water. The size distribution of the NPs is of interest in this study, since TiO_2 NPs are highly hydrophobic; the particles size distribution in water was measured to determine the effect of the stability. The mean size distribution of TiO_2 NP in water was calculated as 432 ± 32 nm for 10 μg/mL NP suspension. Zeta potentials for the TiO_2 NPs in aqueous suspensions were obtained using the Henry Equation. A viscosity of 0.8872 cP, a dielectric constant of 78.5, and Henry function of 1.330 were used for the calculations. The mean zeta potential was calculated as −31.6 ± 4.5 mV at a pH of 7.23 in 10 μg/mL aqueous suspensions of NP.

3.3. Accumulation in Organs of Goldfish. Uptake of nanomaterials by fish and other aquatic species has also been reported previously [13, 33]. This study determined the accumulation



(a) (b)

Figure 1: TEM images for TiO_2 NPs from stock solution (a) and the exposure medium (b).

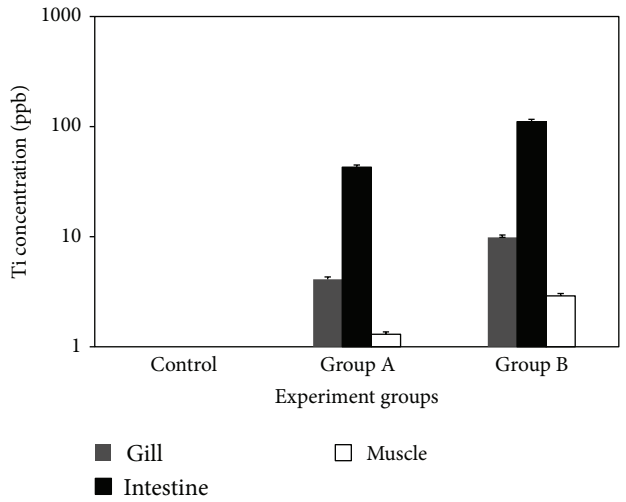


Figure 2: Titanium (Ti) levels in gill, intestine, and muscle of the goldfish at the end of the exposure experiments (Group A and Group B exposed to 10 and 100 mg/L TiO_2 NPs, resp.).

of the unmodified TiO_2 NPs in fish tissues following exposure via the water column without the use of a solvent vehicle or prior modification of the NP surface. The chemical fate of the metal oxide NPs in the aquatic environment was determined through a comprehensive evaluation of uptake in fish with full characterization of the NPs in low and high exposure conditions. The gill, intestine, muscle, and brain of the goldfish were used as *in vitro* model to determine the possible uptake of TiO_2 NPs into tissues. For 10 mg/L and for 100 mg/L TiO_2 exposure mediums, uptakes of TiO_2 NPs in intestine were measured as 42.71 and 110.68 ppb and in gills as 4.10 and 9.86 ppb, respectively. ICP-MS analysis showed very small amount of Ti accumulation in the muscle and no accumulation in brain tissues of the goldfish (Figure 2). A study by Moger et al. [34], however, used coherent anti-Stokes Raman Scattering (CARS) to examine the gills of rainbow trout exposed to 5000 $\mu g\,L^{-1}$ TiO_2 NPs and confirmed the presence of small numbers of particle aggregates within the gill tissue.

3.4. Oxidative Stress. Lipid peroxidation generates a group of products among which are reactive electrophiles such as epoxides and aldehydes [27, 35, 36]. Malondialdehyde (MDA) is a major product of lipid peroxidation in aqueous solution. In this study, to elucidate the possible role of oxidative stress in the effects observed by TiO_2 NPs exposure, MDA content was assayed in liver and muscle of each experimental group. The analysis for MDA content of goldfish liver showed lipid peroxidation in the controls and exposure groups. The mean MDA levels in the liver of the fish were 4.1 ± 0.5, 7.6 ± 1.1, and 11.3 ± 0.9 nmol/gr for control, and low and high dose exposure groups, respectively. No MDA level was measured in the muscles of the control and the exposure groups. Xiong et al. [29] also studied TiO_2 NPs but on zebrafish and reported similar results as this study that oxidative effects were more severe in the livers of zebrafish exposed to 50 mg/L TiO_2 NPs.

Aqueous exposure to low and high dose of TiO_2 NP suspension did not cause any fish mortality during the experimental period (96 hr). Our data is in agreement with the literature, indicating low acute toxicity of TiO_2 NPs to fish survival. Similarly, Warheit et al. [37] reported that the Daphnia magna 48 hr EC50 values and rainbow trout (*Oncorhynchus mykiss*) 96 hr LC50 values for fine TiO_2 particles and ultrafine TiO_2 particles based on nominal concentrations were >100 mg/L, and the LC_{50} for TiO_2 NPs was also found to be over 500 mg L^{-1} in fathead minnow *Pimephales promelas* [38]. Furthermore, Zhu et al. [30] showed that exposure to TiO_2 NPs at the concentrations up to 500 mg/L for 96 hr did not affect hatching rate and did not cause deformity in embryonic zebrafish.

3.5. Effect of NPs on Growth Performance. Out of growth performance parameters for trial groups of goldfish, the best weight-wise growth as of the completion of the trial period was attained in the control group, where NPs were not exposed. The growth of the fish was inhibited with increasing exposure dose of TiO_2 NPs. While the controls showed about 8.1% increase in body weight, those exposed to 10 mg/L NPs showed a small increase (1.8%) in body weight, and those exposed to 100 mg/L showed about 19.7% decrease in body weight at the end of the experiments (Figure 3).

4. Conclusions

A short period of exposure of the waterborne levels of 10 and 100 mg/L TiO_2 NPs is not lethal to the goldfish. Abnormal

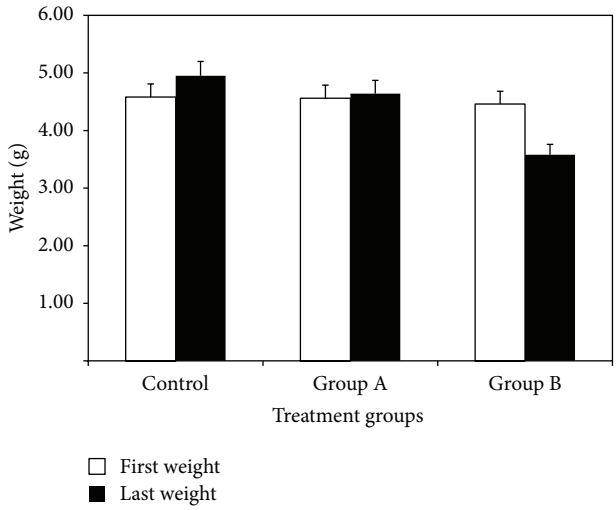


Figure 3: Average live weights (g) for the goldfish at the beginning and at the end of the experiments (Group A and Group B exposed to 10 and 100 mg/L TiO_2 NPs, resp.).

physiological and behavioral changes of the goldfish occurred under the higher concentrations during the experimental period. Since TiO_2 NPs could cause oxidative stress and the decreases in the growth rate on fish, the release of TiO_2 NPs into the aqueous environment may pose potential risks to aquatic organisms. This needs to be considered against the context of a general lack of knowledge of the fate, behavior, and bioavailability of these types of particles in natural systems and suggests a need for longer-term and more environmentally realistic NP exposure regimes to fully determine the transport capabilities of NPs in the aquatic environment. Although data on the behavior and effects of NPs in the environmental food chain would be of primary importance for understanding their overall potential hazard for ecosystems [39], very few studies in fish have examined the uptake and partitioning of TiO_2 NPs, probably due to the difficulties involved in measuring low levels of TiO_2 and limitations in analytical equipment.

Conflict of Interests

The authors declare that they have no conflict of interests.

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