Bioaccumulation of ionic titanium and titanium dioxide nanoparticles in zebrafish eleutheroembryos

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Abstract
The production of titanium dioxide nanoparticles (TiO2 NPs) for commercial applications has greatly increased over the last years and consequently the potential risk for human health. There is a growing awareness of the need to understand the behavior and influence these nanoparticles exert on the environment. Bioaccumulation serves as a good integrator to assess chemical exposure in aquatic systems and is dependent on factors, such as the exposure routes, diet and the aqueous medium. We analyzed the experimental bioaccumulation capability of ionic titanium and TiO2 NPs by zebrafish (Danio rerio) eleutheroembryos through bioconcentration factors (BCFs), after 48 or 72 h of exposure. The stability of both chemical forms in an aquatic medium was fully characterized for further bioaccumulation studies. Several stabilizing agents (humic acids, soluble starch, polyethylene glycol, Na4P2O7 and Na2HPO4) for anatase and rutile, the two allotropes of TiO2 NPs, were evaluated to check the evolution of the aggregation process. Around 60% of TiO2 NPs remained disaggregated under simulated environmental conditions with the addition of 50 mg L−1 of humic acids. However, the presence of eleutheroembryos in the exposure medium increased TiO2 NPs aggregation in the experimental tests. The BCFs values obtained in all cases were <100, which classifies ionic titanium and TiO2 NPs as non-bioaccumulative substances, under the REACH regulations.

Keywords
Aggregation, bioaccumulation, stability, titanium dioxide nanoparticles, zebrafish eleutheroembryos

Introduction
Nanomaterials have great potential for the industry and the commercial sectors with increasing benefits for society. More specifically, titanium dioxide nanoparticles (TiO2 NPs) have gained much importance due to their ability to act as a shield against light and/or their high photocatalytic activity (Gao & Zhang, 2001). The annual production of TiO2 NPs was estimated to be 10 000 tons between 2011 and 2014, and around 2.5 million metrics tons by 2025 (Menard et al., 2011). These NPs are widely used in sunscreens, cosmetics, paints, surface coatings, photocatalysts, ceramic membranes (French et al., 2009; Serpone et al., 2007), and even as a tool for soil, water and air decontamination (Herrmann, 2005). The topical application of products containing TiO2 NPs may lead to their direct or indirect release into the environment (air, soil and water) increasing the risks derived from their presence.

Because of an increased surface-area ratio, NPs have novel specific physical-chemical properties compared to microparticles or even the bulk materials (Egger et al., 2009). It has been acknowledged (e.g. by the OECD chemicals program on cooperation on risk assessment) that the existing risk assessment paradigm developed for traditional chemicals should also be applied to nanomaterials (OECD, 2012), although with specific considerations (e.g. metric to be used, exposure assessment methodology). Several new endpoints in characterization, toxicity and ecotoxicity have been suggested over the last years for a complete evaluation of risk assessment of nanomaterials (ECHA, 2013; OECD, 2010). Bioaccumulation is an important parameter to be considered in risk assessment for chemicals. National and international environmental agencies (US EPA, Environment Canada, German UBA, European ECHA, OECD, etc.) have used it for environmental hazard identification of dissolved chemical species and more recently for NPs (OECD, 2010), for determining the potential for adverse effects on biota (Phillips & Rainbow, 1994). Bioconcentration factor (BCF) is used to measure bioaccumulation for determining when a substance has a potential for long-term environmental impact (Feijtel et al., 1997). Bioaccumulation studies for ionic titanium in adult fish providing a BCF<10 (METI-NITE, 1996) have shown the non-bioaccumulative characteristic of this chemical form. However, there is scarce information regarding TiO2 NP bioaccumulation in aquatic organisms since the range of BCF already published is expanded. Zhu et al. (2010a) reported bioconcentration factors of up to 118 062 L kg−1 for TiO2 NPs in Daphnia magna when exposed to 1 mg L−1 of TiO2 NPs for 72 h. In another study with rainbow trout (Oncorhynchus mykiss), exposed to concentrations up to 1 mg L−1 of TiO2 NPs for 14 days, no bioaccumulation of TiO2 NPs was determined (Federici et al., 2007). Further evaluation of TiO2 NPs bioaccumulation capacity is required to clarify the existing results.

Several studies have shown the aggregation capability of TiO2 NPs in aqueous environments (French et al., 2009; Von der Kammer et al., 2010). Once released into the aquatic...
environment, TiO$_2$ NPs tend to aggregate rapidly forming larger particles. Particles can then form sediments or be transported as stable particles for long distances. Factors, such as the pH, ionic strength, presence of ligands in natural waters, etc., can affect the mobility, persistence, bioavailability and reactivity of these particles. For example, natural organic matter, ubiquitous in natural waters, has shown to significantly affect the stability of certain NPs in the aquatic environment (Gao et al., 2012; Li & Sun, 2011). Humic acids protect and inhibit TiO$_2$ NP aggregation through electrostatic repulsion by modifying their surface properties (e.g. electric charge, size or chemical nature of the exposed surface sites) (Domingos et al., 2009, Xiong et al., 2011).

One of the main requirements of all standard bioaccumulation tests is to maintain a constant concentration of the tested compound. Regarding NPs, particularly TiO$_2$ NPs, their concentration can change in water, because they tend to agglomerate easily and precipitate under certain conditions. It is important to establish the conditions at which TiO$_2$ NPs are stable. Capping and/or stabilizing agents are usually employed to prevent their aggregation. Different stabilizers can be used for TiO$_2$ NPs; those compatible with aquatic life are the following: polyethylene glycol (PEG), used for coating TiO$_2$ NPs in biomedical applications; PEG increases their biocompatibility and efficiency (Devanand Venkatasubbu et al., 2013); soluble starch, a green capping agent (Vasileva et al., 2011); Na$_2$P$_2$O$_7$ and Na$_2$HPO$_4$, electrolytes that increase the electrostatic repulsion between NPs or between NPs and surface particles (Von der Kammer et al., 2010); and humic acids (HA), which inhibit TiO$_2$ NP aggregation in aqueous environments by electrostatic repulsion (Domingos et al., 2009).

Here, we separately tested the aggregation of two allotropic forms of TiO$_2$ NPs (anatase and rutile) in an exposure media with different stabilizers compatible with aquatic life. Anatase and rutile have different surface properties and reactivity, and they induce different types of adverse effects. Rutile has lipophilic properties, whilst anatase has hydrophilic properties (Clément et al., 2013). We studied them separately trying to maintain a size range below 100 nm. Furthermore, the characterization of a mixture of anatase and rutile TiO$_2$ NPs (75%:25%), commonly used for toxicological assays, was also carried out. To assess the bioaccumulation of TiO$_2$ NP by zebrafish eleutheroembryos as a vertebrate model system, a bioconcentration test previously employed with other chemical compounds (Cuello et al., 2012; El-Amrani et al., 2012; Gonzalo-Lumbrares et al., 2012; López-Serrano et al., 2011) and NPs (López-Serrano et al., 2014) was used in this study. The BCFs were calculated using a biodynamic model proposed by Spacie & Hamelink (1985). To understand the differences in behavior between ionic titanium and TiO$_2$ NPs, bioaccumulation of ionic titanium was also assessed.

Methods

Reagents, instruments and apparatus

Analytical grade chemicals were used for all experiments. Ionic titanium solution (1000 mg L$^{-1}$ Ti, TraceCert®, Sigma-Aldrich, Chemie, GmbH 30918, Seelze, Germany) was used to prepare the standard solution. Diluted solutions were prepared from a stock solution using Milli-Q Element ultrapure water (Millipore, Billerica, MA). All ionic titanium solutions were prepared by adding citric acid up to 0.1% (m/V) to avoid precipitation at the mg L$^{-1}$ level. Nitric acid (HNO$_3$, 65%), hydrofluoric acid (HF, 47–51%) (Merck, Darmstadt, Germany), hydrogen peroxide (H$_2$O$_2$, 35%) and boric acid (H$_3$BO$_3$) (Panreac, Barcelona, Spain) were used to digest TiO$_2$ NPs from the eleutheroembryos. Exposure ISO solution (ISOwater) of similar composition to fresh river water was prepared as follows: 294 mg of CaCl$_2$.2H$_2$O; 123.3 mg of MgSO$_4$.7H$_2$O, 63 mg of NaHCO$_3$ and 5.5 mg of KCl were diluted to 1 L with distilled water (ISO, 1996). Standard dispersions were prepared using rutile and anatase TiO$_2$ NPs purchased from Sigma Aldrich (Germany). Titanium (IV) oxide, anatase, nanopowder, <25 nm particle size, 99.7% trace metals (CAS: 1317-70-0), and rutile, nanopowder, <100 nm particle size, 99.5% trace metals (CAS: 1317-80-2). The tested stabilizers were: soluble starch GR (Merck); polyethylene glycol, PEG, (Aldrich); humic acids (Sigma Aldrich), Na$_3$P$_2$O$_7$ (Sigma Aldrich) and Na$_2$HPO$_4$ (Panreac).

A sonication bath (Ultrasons-HD 10 Liters: Selecta, Barcelona, Spain) was used to prepare the assayed TiO$_2$ NPs dispersions. A Vibra cell VC × 130 (SONICS Vibra CellVCX130, Newtown, CT) focused ultrasonic probe (USP) (Connecticut, USA) equipped with a 3-mm-diameter titanium micropipet and fitted with a high-frequency generator of 130 W at a frequency of 20 kHz was used for sample treatment. A centrifuge model FVL-2400 N from Combi-Spin (Boco, Germany) was used for sample centrifugation during experimentation. Inductively coupled plasma mass spectrometer ICP-MS HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan) equipped with a Babington nebulizer, Fassell torch and double-pass Scott-type spray chamber cooled by a Peltier system was employed to determine total titanium content. Single ion monitoring at m/z 48 was selected for data collection. Electron microscopy studies to evaluate the state of TiO$_2$ NPs in the exposure media were performed using a JEOL JEM 3000FX (Tokyo, Japan) Transmission Electron Microscope (TEM) equipped with a microanalysis system (Oxford Instruments, Tubney Woods, Abingdon, Oxfordshire, UK).

Anatase/Rutile NP preparation

Stock suspensions of 1000 mg L$^{-1}$ of anatase, rutile, and the 75% anatase:25% rutile mixture in Milli-Q water were prepared by adding the TiO$_2$ NP powder in deionized water, followed by ultrasonication for 30 min. Dilution of these suspensions from 1000 mg L$^{-1}$ TiO$_2$ NPs with the ISOwater media after adding the corresponding stabilizers at different concentrations was performed. Sonication was next performed for 30 min with the ultrasonic bath to avoid possible aggregation of the NP.

Characterization of TiO$_2$ NP stability

To determine the type of stabilizer that best maintains TiO$_2$ NPs as NPs in the exposure media, several suspensions at 2 mg L$^{-1}$ of TiO$_2$ NPs were prepared in the following media: (i) 0.1–0.2% w/v of soluble starch; (ii) 0.1% w/v of chitosan; (iii) 0.1% w/v of PEG; (iv) 2.5–100 mg L$^{-1}$ of humic acids; (v) 1–5 mM of Na$_3$P$_2$O$_7$; and (vi) 0.3% w/v of Na$_2$HPO$_4$. All these suspensions were analytically characterized by ultrafiltration as previously described (López-Serrano et al., 2014). Briefly, 100 mL of the suspension was prepared (fraction 0); next, 2 mL of that suspension was filtered through a 100 nm pore size membrane, obtaining fraction 1; the same procedure was repeated using 50 and 25 nm pore size membranes, obtaining fraction 2 and fraction 3, respectively. Titanium content in each fraction was determined by a flow injection system coupled to an ICP-MS detector; each determination was performed in triplicate.

TiO$_2$ NP size measurement results obtained by the ultrafiltration experiments were also validated through TEM. Homogeneity of TiO$_2$ NP suspensions dissolved in the ISOwater plus 50 mg L$^{-1}$ of humic acids (HA), at the desired concentration, was achieved by sonication for 30 min with (a) focused ultrasonic probe (USP), (b) ultrasonic bath and (c) ultrasonic bath and subsequent freezing of the sample. For the measurements, two drops of each sample were deposited over a copper grid.
Exposure of eleutheroembryos

Eleutheroembryos were obtained from wild type adult zebrafish bred and maintained in the AZTI Zebrafish Facility (EU-10-B1) under standard conditions. All experimental procedures were approved by the Regional Animal Ethics Committee. Five tanks were used to perform experiments: one for the control (free from any of the target contaminants) and one for each of the different concentrations of the compounds to be tested (two with ionic Ti and two with TiO2 NPs). The nominal concentrations used for the exposure were selected based on the OECD test 305 (OECD, 1996). According to this test, the highest concentration should be around 1% of the LC50 value of the compound and the second concentration should differ by a factor of 10. For TiO2 data in the literature indicate a 96-h LC50 value above 500 μg L⁻¹ for D. magna (Heinlaan et al., 2008) and a 48-h LC50 > 20 mg L⁻¹ for TiO2 for medaka (Oryzias latipes) (METI-NITE, 2006). The nominal concentrations chosen for this study were 0.1 and 1 mg L⁻¹ for ionic titanium and 2 and 10 mg L⁻¹ for TiO2 NPs.

Solutions of ionic titanium were prepared as explained in experimental procedures by adding citric acid up to 0.1% to prevent precipitation, and then adjusting pH to 6.8 for larvae survival. The choice of these concentrations is based on the LC50 > 100 mg L⁻¹ reported in fish (124.5 mg L⁻¹ for fish embryos (Xiong et al., 2011) and >100 mg L⁻¹ for trout (Oncorhynchus mykiss) (Warheit et al., 2007).

To characterize the bioaccumulation of titanium as ionic and as TiO2 NPs in the exposure media and compare the BCFs, an appropriate number of eleutheroembryos after 72 hpf (hours post fertilization), time at which embryos become eleutheroembryos, were transferred to 1 L tanks filled with ISOwater spiked with the selected concentrations of ionic titanium and TiO2 NPs. TiO2 NPs were previously treated with 50 mg L⁻¹ of HA to avoid their aggregation in the standard aqueous media, as described above. Exposures were done at 27°C with a 12-h photoperiod. The exposure test consisted of two phases: (a) absorption, 48 h or 72 h in a contaminated exposure medium, and (b) depuration, 24 h in a clean exposure medium. Around 20 eleutheroembryos were collected from the tanks at the different times to determine analyte concentration. According to the OECD Test 305, the eleutheroembryon loading rate at the beginning of the experiments should range between 0.7 and 0.8 g L⁻¹ (wet weight) and the observed mortality should be lower than 20% at the end of the test. Sampling times were as follows: t0, t3h, t6h, t21h, t45h, t48h. Additional eleutheroembryos were left up to 72 h in the uptake phase and up to 96 h in the depuration phase for both chemical forms.

Quantification of titanium by ICP/MS

The USP was employed for leaching the analytes from the eleutheroembryos after the incubation period in the exposure media spiked with ionic titanium. 1000 μL of HNO3 (6.5%) were added to promote the leaching of titanium from zebrafish eleutheroembryos exposed to ionic titanium. Eleutheroembryos exposed to TiO2 NPs were digested with 0.3 mL of HNO3 (65%), 0.15 mL of concentrated HF (47–51%), 0.15 mL of H3BO3 (0.1% w/v), and 0.05 mL of H2O2 (35%). Titanium was determined by a flow injection system coupled to an ICP-MS (FIA-ICP-MS). Titanium determined in the exposure media was averaged from two different replicates. For zebrafish eleutheroembryos, three analytical replicates (consisting of 20 individual each) were measured.

Bioaccumulation kinetics and statistics

To assess the bioaccumulation by zebrafish eleutheroembryos of both titanium chemical forms, BCFs were determined following the OECD guidelines test 305 (OECD, 1996). Total titanium concentration in eleutheroembryos and exposure solution at 48 h of accumulation (BCF48h) was quantified according the BCF classical definition (Feijtel et al., 1997). When a steady state is not reached, BCF values can also be calculated from a first-order two-compartment (water and aquatic organism) model (Gobas & Zhang, 1992; Spacie & Hamelink, 1985); this has been previously used (El-Amrani et al., 2012; López-Serrano et al., 2014) and described in Supporting Information (SI). The software NONLIN 5.1 (Nashville, TN) was used for the kinetic calculations (Sherrod, 1995). Bioconcentration factors were calculated applying the two different procedures to the experimental data, obtaining BCF48h and BCFk.

Results

TiO2 NP characterization in the exposure media

Ultrasiltration results using different stabilizers for each allotropic structure to avoid NP aggregation and/or dissolution in the ISOwater media used for the bioaccumulation tests are shown in Table 1. These results revealed that total titanium

| Capping agent | 100 nm > NPs > 50 nm | 50 nm > NPs > 25 nm | 25 nm > NPs | 100 nm > NPs > 50 nm | 50 nm > NPs > 25 nm | 25 nm > NPs |
|---------------|----------------------|---------------------|------------|----------------------|---------------------|------------|
| None          | 2 ± 1                | 3 ± 1               | 7 ± 1      | 7 ± 1                | ND                  | ND         |
| 0.1% starch   | 16 ± 1               | ND                  | ND         | 8 ± 1                | 7 ± 1               | ND         |
| 0.2% w/v starch| 11 ± 1               | 12 ± 2              | 2 ± 1      | 16 ± 1               | 10 ± 2              | ND         |
| 0.1% w/v chitosan| ND                  | ND                  | ND         | ND                  | ND                  | ND         |
| 0.1% w/v PEG | 3 ± 1                | 2 ± 1               | 3 ± 1      | 5 ± 1                | 6 ± 1               | 3 ± 1       |
| 2.5 mg L⁻¹ HA | 3 ± 1                | 2 ± 1               | 2 ± 1      | 2 ± 1                | 3 ± 1               | 3 ± 1       |
| 20 mg L⁻¹ HA | 12 ± 1               | 1 ± 1               | ND         | 80 ± 5               | 20 ± 2              | 2 ± 1       |
| 50 mg L⁻¹ HA | 64 ± 4               | 15 ± 1              | 17 ± 3    | 44 ± 1               | 6 ± 1               | 5 ± 1       |
| 100 mg L⁻¹ HA| 44 ± 1               | 6 ± 1               | 5 ± 1      | 18 ± 1               | 5 ± 2               | 5 ± 1       |
| 1 mM Na2P2O7  | 32 ± 3               | 3 ± 1               | 2 ± 1      | 2 ± 1                | ND                  | ND         |
| 2.5 mM Na2P2O7| 40 ± 5               | 6 ± 1               | 2 ± 1      | 21 ± 4               | 3 ± 1               | 2 ± 1       |
| 5 mM Na2P2O7  | 110 ± 16             | 4 ± 3               | 4 ± 1      | 92 ± 3               | 7 ± 1               | 3 ± 1       |
| 0.3 % w/v NaH2PO4| 56 ± 1              | ND                  | ND         | 6 ± 1                | ND                  | ND         |

Table 1. Ultrafiltration results to establish particle size distribution using different stabilizers to maintain TiO2 NPs <100 nm.

Recovery expressed as % represents total titanium determined in the initial suspension (before ultrafiltration), considered 100%. [TiO2 NPs] = 2 mg L⁻¹.
in Fraction 1 was below 20% with most assayed stabilizers; the two best stabilizers were: (a) 5 mM Na₄P₂O₇, providing an excellent non-aggregation state for both anatase and rutile, and (b) 50 mg L⁻¹ humic acids (HA) that keep about 60 and 40% of anatase and rutile TiO₂ NPs, respectively, below 100 nm. However, the mortality rate of zebrafish larvae at 5 mM of Na₄P₂O₇ was almost 100% after 24-h of exposure. For this reason, HA was chosen as the capping agent for subsequent bioaccumulation studies. It is noteworthy that total titanium in Fraction 3 under these conditions was below 10%, indicating that the dissolution of TiO₂ NPs in the exposure media was negligible.

Particle size distribution was characterized for each sampling time (0 to 72 h of exposure) by ultrafiltration, demonstrating that aggregation increases with time and especially in the presence of zebrafish eleutheroembryos. During this time, around 30% of total TiO₂ NPs maintained a size <100 nm. These ultrafiltration results showed an aggregation slightly higher than the expected, suggesting that the presence of eleutheroembryos can affect the behavior of TiO₂ NPs in the exposure media. This finding also indicates that the exposure of eleutheroembryos to TiO₂ NPs as aggregates represents a more environmentally realistic scenario and could constitute the basic of different bioaccumulation patterns of NPs against ionic titanium.

TEM results were consistent with those obtained following the ultrafiltration procedure. TiO₂ NPs prepared in IS0water with 50 mg L⁻¹ HA appeared as spherical individual particles forming aggregates (Figure 1a–c). Sizes of TiO₂ NP aggregates within the solutions ranged between 50 and 300 nm depending on the ultrasonic device applied. This means that eleutheroembryos can be heterogeneously exposed to different surface areas and proportions of TiO₂ NPs. TEM images also demonstrated how the storage temperature is a crucial parameter that affects the aggregation rate of the NPs: significant aggregation of TiO₂ NPs (larger than 300 nm) was seen when samples were stored at −20°C (Figure 1d).

### Bioaccumulation experiments

#### Titanium quantification in the exposure medium and in eleutheroembryos

Titanium content determined in the exposure media at different sampling times for both ionic and NP experiments (Figures 1 and 2 at SI) was in line with the OECD Test Guideline 305, where a maximum variation of 20% from nominal concentration is allowed. Total titanium concentration measured in the exposure media, in the eleutheroembryos used as control, and in exposure media during the depuration phase was negligible.

Bioaccumulation of ionic titanium in eleutheroembryos increased with the exposure time, reaching an incipient steady state at around 48 h (Figure 2a), which was confirmed by extending the uptake phase to 72 h (Figure 2b). The concentration of titanium in eleutheroembryos during the depuration phase drastically decreased in less than 24 h. Bioaccumulation of ionic titanium in eleutheroembryos also increased over time when exposed to TiO₂ NPs (Figure 3), rapidly decreasing over the depuration phase, similar to what was observed for ionic titanium. Thus, eleutheroembryos were able to eliminate the titanium very quickly regardless of the chemical form they accumulate.

#### Bioconcentration factors

Table 2 shows the bioaccumulation kinetics values obtained for ionic titanium and TiO₂ NPs, as well as the concentration values in the exposure medium (Cₜ), in eleutheroembryos (Cₑ), the uptake and depuration rate constants (k₁, k₂), and the bioconcentration factors (BCF₄₈ and BCFₖ). BCF values obtained were 77 and 3 at 0.1 and 0.9 mg L⁻¹ nominal values for ionic titanium and 4.3 and 3 at 2 and 10 mg L⁻¹ nominal values for TiO₂ NPs, respectively. Table 1 in SI summarizes the statistical data of the fit to the models. According to the REACH regulations, a substance is considered as bioaccumulative if the BCF >2000 and very bioaccumulative if the BCF >5000 (EC, 2006). Thus, the bioconcentration factors obtained for all the experiments classify

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**Figure 1. TEM image of TiO₂ NPs (Anatase 75%; Rutile 25%).** (a) and (b) samples were treated in an ultrasonic bath; (c) sample treated with an ultrasonic focused probe; (d) sample stored at −20°C and thawed at the time of the analysis. Capping agent: 50 mg L⁻¹ HA.
ionic and TiO$_2$ NPs as non-bioaccumulative by zebrafish eleutheroembryos when the exposure occurs in water.

**Discussion**

**TiO$_2$ NPs characterization and stability in the exposure media**

Ultrafiltration results highlight the tendency of TiO$_2$ NPs to aggregate in most of the tested aqueous media, which is in line with results published elsewhere (French et al., 2009; Von der Kammer et al., 2010; Xiong et al., 2011; Zhu et al., 2010b). NP aggregation can affect TiO$_2$ NP bioaccumulation because of a reduced mobility, different interactions with filtering and sediment-eating organisms, or even with suspended organic matter (Auffan et al., 2009; Maurer-Jones et al., 2013). Here, several stabilizers, commonly used to prevent TiO$_2$ NPs aggregation, were tested to get more stable colloidal solutions and facilitate the exposure to aquatic organisms. The addition of 50 mg L$^{-1}$ of HA, usually present in natural waters, maintained an important percentage of total TiO$_2$ NPs as ultrafine NPs in the case of anatase and rutile. HA decreased TiO$_2$ NP aggregation by increasing the electrostatic repulsion of anatase and rutile NPs (Phenrat et al., 2010). However, an increased aggregation rate was observed for the 75% anatase/25% rutile mixture. The combination of both allotropic structures, with quite different surface and reactivity properties, could cause a higher interaction rate between particles, preventing the binding of HA to the surface of the NPs.

Here, we have showed that when exposed to environmental concentrations of HA (Akaighe et al., 2011), TiO$_2$ NPs have a high tendency to form aggregates larger than 100 nm, which is in line with other studies (Lin et al., 2012; Sillanpää et al., 2011). Lin et al. (2012) communicated that the hydrodynamic size of TiO$_2$ NPs decreases with increased NP surface-bound HA.
concentration, determining that the lowest TiO$_2$ NP hydrodynamic diameter was 510$\pm$20 nm at the highest tested HA concentration, 1500 mg L$^{-1}$. Li & Sun (2011) found a high degree of TiO$_2$ NPs aggregation with a nominal size of 30 nm in aqueous suspensions; TiO$_2$ NPs were present in aqueous suspensions as micron-sized aggregates, although in the presence of fulvic acids aggregate size reduction was seen. Aggregation was also dependent on pH, TiO$_2$ NPs exhibited a high degree of aggregation at higher pH (Li & Sun, 2011).

The presence of eleutheroembryos favors TiO$_2$ NPs aggregation in aqueous media. The presence of aquatic organisms modifies the bioavailability of NPs from aqueous media as pointed out by other authors, e.g. D. magna had an effect on the aggregation of natural colloid minerals (Filella et al., 2008). This indicates the importance of monitoring NP size distribution during this type of studies. Johnston et al. (2010) also observed that metallic oxide NPs formed aggregates and precipitated in presence of fish, decreasing their bioavailability. Furthermore, the existence of aquatic organisms in the aqueous media could decrease the fraction of HA available to bind to the surface of the TiO$_2$ NPs. HA may attach to the surface of the eleutheroembryos, which would lead to an increase in the aggregated TiO$_2$ NP fraction leading to deposition at the bottom and therefore decreasing their bioavailability. This fact emphasizes the importance of understanding the behavior and fate of NPs regarding bioavailability to aquatic organisms.

Predicting the chemical behavior of NPs in the environment is quite difficult. Many factors contribute to their stability and behavior; structure, composition and surface chemistry of the particles, water chemistry, etc. Here, simulated realistic conditions (standard ISOwater media, with a salt composition similar to that found in natural waters, 50 mg L$^{-1}$ of HA, and presence of zebrafish eleutheroembryos) caused the formation of TiO$_2$ NPs aggregates larger than 100 nm. Conditions of this more realistic scenario should be considered to assess the adverse effects of NPs to aquatic organisms in the environment.

**Titanium bioaccumulation by zebrafish eleutheroembryos**

In this study, we have shown that both ionic titanium and TiO$_2$ NPs accumulate in zebrafish eleutheroembryos over time. Furthermore, zebrafish eleutheroembryos can eliminate over 50% of the total accumulated titanium in less than 24 h. This tendency was independent of the titanium chemical form or the exposure concentration. According to REACH (EC, 2006), the BCF values obtained in this work indicate there is no significant bioaccumulation of ionic titanium and TiO$_2$ NPs by zebrafish eleutheroembryos.

Some of the experimental data obtained for ionic titanium fitted poorly with the bioaccumulation model (Figure 2a and b). Data from the first hours of the experiment and the depuration phase distorted the model distribution, and were not considered in the overall analysis in order to obtain more consistent results from both experimental trials. Despite this, the BCF values obtained in our experiments (3–77) are in agreement with the few previous studies published. The METI-NITE Japanese database listed BCF values within the range 1.1–10 when the carp Cyprinus carpio specimens were exposed to 2 and 0.2 mg L$^{-1}$ of dissolved titanium dioxide (METI-NITE, 2006). In another study, adult zebrafish (D. rerio) were exposed to 1 mg L$^{-1}$ of bulk TiO$_2$ and a BCF of 7.2 was determined (Ramsden et al., 2013). The different concentrations and biological models used in the various studies could explain the contrasting BCFs values. An inverse relationship between the BCFs and the exposure concentration has also been reported (Cuello et al., 2012; McGeer et al., 2003). Bioaccumulation mechanisms are dependent on the nature of the tested compound: while passive diffusion across the lipid bilayer of biological membranes, as predicted by Fick’s Law, is the main process for neutral organic substances, the bioaccumulation of low lipophilicity metals in biota differs from that of neutral organic molecules. The uptake of metals occurs by complex dynamics (specific channels in the cell membrane, active transport or endocytosis), they are stored in detoxified forms, such as inorganic granules or bound to metallothionein-like proteins, active elimination, etc. (Simkiss & Taylor, 1989). Other authors point out the importance of the composition of the surrounding media during metal accumulation (Komjarova & Blust, 2009). Bioconcentration factors neither distinguish between essential mineral nutrient, normal background metal concentration, the capabilities of animals to vary uptake and elimination depending on exposure concentrations, nor the specific ability to sequester, detoxify and store internalized metals from metal uptake that results in adverse effect. All these explain why BCFs values are dependent on the concentration of the exposure media (McGeer et al., 2003).

The experiments with TiO$_2$ NPs were carried out using a 72-h exposure period since previous experiments showed a non-stationary stage using only 48 h for bioaccumulation. The same effect was found by other authors for D. magna exposed to TiO$_2$ NPs with minimal bioaccumulation within the traditional 48-h exposure time, only increased when the exposure time was extended to 72 h (Zhu et al., 2010a). BCF values for TiO$_2$ NPs already published in the literature is broad, from 118.063 to no bioaccumulation (Federici et al., 2007; Scown et al., 2009) (Table 3). The data communicated for D. magna (Zhu et al., 2010a) is quantitatively different from our results; this species is a crustacean and the uptake and depuration process can substantially differ from that of fish. Reported BCF data for adult carps, ranging between 325 and 617, was obtained on dry weight basis (FAO, 2013). These BCF values should be corrected for proper comparison. Thus, two main sets of data for BCF values, one of approximately 100 and another group of around 1 or no bioaccumulation, have been reported. The presence of humic acids and the formation of aggregates could decrease the bioavailability of TiO$_2$ NPs to zebrafish eleutheroembryos resulting in a decrease of their bioaccumulation. Lin et al. (2012) showed that HA bound to the surface of TiO$_2$ NPs prevents the adhesion of TiO$_2$ NPs to algal cells due to an increased electrostatic repulsion. As we have stated before, the composition of the media can affect the size of the NPs, and consequently their bioavailability and bioaccumulation.

**Conclusions**

The results presented here allow us to classify ionic titanium and TiO$_2$ NPs as non-bioaccumulative substances by zebrafish eleutheroembryos after water exposure according the REACH regulations (EC, 2006). Although new data, documents and regulations about NPs are generated continuously, current guides for risk assessment still use the same protocols and methodologies for NMs as for other substances (i.e. OECD 305 for bioaccumulation or OECD 203 for acute toxicity). The BCF values obtained using zebrafish eleutheroembryos are relatively in good agreement with the average data published. Therefore, considering the need of additional experiments, these results together with other already published and cited in this paper show that this model could be a promising approach for calculating bioconcentration factors. Our method would cheapen the assays by using zebrafish larvae instead of adult fish because of the minor maintenance of larvae, the lower time needed to complete the study, and the smaller amount of chemicals needed. Data found in the literature on TiO$_2$ NPs bioaccumulation include a wide range of BCFs
The special physicochemical properties of the NPs. Furthermore, new methods for rapid NP detection in fish tissues and NP characterization through these ecotoxicological tests should be developed. Further studies to assess the role of the physicochemical properties of NPs on fish muscle and/or organ bioaccumulation are required.

**Declaration of interest**

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. Authors confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and there are no impediments to publication, including the timing of publication, with respect to intellectual property. In doing so, we confirm that we have followed the regulations of our institutions concerning intellectual property.

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values, dependent on the exposure period and the studied living organism. The agglomeration state of TiO2 NPs must always be considered, as it may affect the uptake limit or prevent bioaccumulation by the zebrafish eleutheroembryos when the only exposure route is water. We have shown that the presence of humic acids is a suitable approach to stabilize TiO2 NPs as they significantly decrease the tendency of TiO2 NPs to agglomerate, are innocuous to living organisms, and good simulators of environmental waters. Data provided here and published elsewhere indicate the need for new standardized ecological bioaccumulation test protocols that take into account the special physicochemical properties of the NPs. Furthermore, new methods for rapid NP detection in fish tissues and NP characterization through these ecotoxicological tests should be developed. Further studies to assess the role of the physicochemical properties of NPs on fish muscle and/or organ bioaccumulation are required.

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Supplementary material available online

Supplementary Table 1 and Supplementary Figures 1–2.