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RESEARCH ARTICLE

Lethal and sub-lethal effects of nanosized titanium dioxide particles on *Hydropsyche exocellata* Dufour, 1841

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ABSTRACT

Nano-sized titanium dioxide particles (nTiO₂) are one of the most widely used nano-scale engineered materials. Evidence of the presence of nTiO₂ in freshwater systems has been reported by recent ecotoxicological studies, which indicated the potential toxic effects of nTiO₂ to aquatic organisms. This study aimed at exploring lethal and sub-lethal endpoints of aqueous exposure of the aquatic insect *Hydropsyche exocellata* Dufour, 1841 (Trichoptera, Hydropsychidae) to nTiO₂ at different concentrations. Abdominal contractions significantly increased with nTiO₂ exposure. Levels of lipid peroxidation (LPO) increased with nTiO₂ dose, whereas the opposite was recorded for the antioxidant enzyme catalase activity (CAT). Moreover, the highest exposure (50.0 mg/L) resulted in the highest mortality (56% of mortality two weeks after pulse exposure to nTiO₂). Overall, our results show that nTiO₂ has potential toxic effects on *H. exocellata* at concentrations of 50.0 mg/L.

KEYWORDS: Trichoptera; nanoparticles; toxicity; image processing; biomarkers

Introduction

Nanomaterials have been commercialised for decades, but the pace seems to be accelerating (Chen, Liu, Zhang, Ying, and Jin 2011), with up to 1300 referenced products containing nanomaterials (Dobias and Bier-Latmani 2013). Due to the increasing production volumes, an environmental exposure to engineered nanomaterials (ENM) is basically inevitable (Nowack and Bucheli 2007). Several ecotoxicological studies have reported some data about the effects of nanoparticles on the aquatic organisms (e.g., Campos, Rivetti, Rosenkranz, Navas, and Barata 2013; Canesi et al. 2012; Faria, Navas, Soares, and Barata 2014; Wang, Wick, and Xing 2009; Zhu and Cheng 2010). Gottschalk, Sonderer, Scholz, and Nowack (2009) predicted environmental concentrations of ENM based on a probabilistic material flow analysis from a life-cycle perspective of ENM containing products and indicated that risks to aquatic organisms may currently emanate from nano-Ag, nano-TiO₂, and nano-ZnO discharged from sewage treatment effluents (Gottschalk et al. 2009). Manufactured nano-scale titanium dioxide particles (nTiO₂) are one of the most popular ENMs, being used in a broad range of consumer products including sunscreens, cosmetics, paints and other coatings, and embedded in glass and other surfaces (Fisher and Egerton 2001; Kaida, Kobayashi, Adachi, and Suzuki 2004). TiO₂ nanoparticles are also applied in

wastewater treatments (Chen et al. 2004) and environmental remediation (Aitken, Chaudhry, Boxall, and Hull 2006). Direct evidence for the release of synthetic nTiO₂ from urban sources into the aquatic environment has been already reported (Kaegi et al. 2008; Kiser et al. 2009). For example, a high nTiO₂ concentration ($3.5 \cdot 10^8$ particles/L) was found by Kaegi et al. (2008) in runoff water that contacted painted facades. Therefore, nTiO₂ could pose a risk to the aquatic environments, particularly freshwater and coastal environments, because of their proximity to human populations and industry and wastewater discharges (Ward and Kach 2009). Gottschalk et al. (2009) predicted nTiO₂ environmental concentrations of 0.015 µg/L as the most frequent concentration in surface waters for different regions of Europe. From an ecological point of view, these concentrations are not currently of concern, but the problem lies in the increasing uses of ENM over time that could produce an increasing level of nTiO₂ environmental concentrations in the future.

Generally, the toxicity of nanoparticles is associated with their small size and high specific surface area reactivity (Lovern and Klaper 2006), being theoretically more toxic than their bulk counterparts. Their reduced size allows them to penetrate into and accumulate within cells and organisms (Carlson et al. 2008; Ispas et al. 2009; Mironava, Hadjiargyrou, Simon, Jurukovski, and Rafailovich 2010). Uptake of nanoparticles in aquatic animals is likely to be by direct passage across gill and other external epithelia (Moore 2006) because of their greater surface reactivity with animal tissues. Additionally, the ingestion of contaminated food can be a substantial route of exposure, such as coated algae ingested by aquatic organisms (Cedervall, Hansson, Lard, Frohm, and Linse 2012). There is an increasing number of studies that underline the potential risk of nanoparticle exposure to aquatic life in the water column and sediment compartments (e.g., Blaise, Gagné, Férard, and Eullaffroy 2008; Canesi et al. 2012; Federici, Shaw, and Handy 2007; Lovern and Klaper 2006; Lovern, Strickler, and Klaper 2007; Oberdörster 2004; Smith, Shaw, and Handy 2007; Warheit et al. 2007). These studies include a great diversity of nanoparticles (e.g., TiO₂, CdSe, Au, ZnS, ZnO, Ag, Polystyrene, SiO₂, CuO, Fe₃O₄) and aquatic species from different trophic levels: such as algae (Bour, Mouchet, Silvestre, Gauthier, and Pinelli 2015), clams (Buffet et al. 2013; Renault et al. 2008), daphnids (Bouldin et al. 2008; Fouqueray et al. 2012; Zhao and Wang 2010; McTeer, Dean, White, and Pittman 2014), snails (Croteau, Dybowska, Luoma, and Valsami-Jones 2011a; Croteau, Dybowska, Luoma, and Valsami-Jones 2011b), copepods (Jarvis, Miller, Lenihan, and Bielmyer

2013), amphipods (Jackson, Bugge, Ranville, and Chen 2012) and fish (Bour et al. 2015). However, most of these organisms are related with lentic environments (e.g., lakes, wetlands and ponds), limiting our knowledge on the potential impact of nanoparticles on rivers and streams.

The effects of nTiO₂ exposure on aquatic biodiversity have been mostly reported by using *Daphnia magna* Straus, 1820 (Cladocera) as a model organism (Adams, Lyon, McIntosh, and Alvarez 2006; Campos et al. 2013; Hund-Rinke and Simon 2006; Lovern and Klaper 2006; Lovern et al. 2007; Warheit et al. 2007; Zhu and Cheng 2010). *Daphnia magna* is generally the food and energy link between the primary producers (algae) and secondary consumers (fish and fish larvae) in lentic environments. However, there is lack of toxicity tests using aquatic insects, which play an equivalent ecological role to *D. magna* in lotic environments (i.e., rivers and streams). Furthermore, available studies have focused on acute or chronic exposures to nTiO₂ but there is no information on pulse exposures, which could occur when ENMs are released from a hypothetical accidentally municipal wastewater effluent to discharge to rivers and streams (Baker et al. 2016). In our study, we used the net-spinning caddisfly *Hydropsyche exocellata* Dufour, 1841 (Trichoptera, Hydropsychidae) as a model organism to evaluate the potential toxic effects of nTiO₂ pollution on lotic organisms. This species is widespread in Western Europe (Bonada, Zamora-Munoz, Rieradevall, and Prat 2004) and it plays a key role in rivers and streams by capturing suspended particles (Wallace, Webster, and Woodall 1977) and serving as food for fish (Cadwallader 1975; Elliott 1967). To our knowledge, there is no data available on the toxic effects of nanoparticles on this species, which constitutes an excellent model system due to its ability to adapt to laboratory conditions and to its wide variety of easily recordable sub-lethal responses to toxicity, such as, locomotion (Gerhardt 1996; Macedo-Sousa, Gerhardt, Brett, Nogueira, and Soares 2008; Sala et al. 2016), the symmetry of the nets that it builds to capture food particles (Besch, Schreiber, and Magnin 1979; Petersen and Petersen Jr 1984; Tessier, Boisvert, Vought, and Lacoursiere 2000) and biomarkers (Barata, Lekumberri, Vila-Escalé, Prat, and Porte 2005; Sala et al. 2016). Sub-lethal effects are important to consider because they can serve as early-warning signals of pollution that alert us before the risk of damage to the ecosystem is too high (Gerhardt 1996; Ren, Zha, Ma, Wang, and Gerhardt 2007).

The aim of this study was to explore the toxic effects induced by nTiO₂ on *H. exocellata* larvae, by using a pulse exposure scenario at different concentrations (0.5

mg/L; 1.0 mg/L; 5.0 mg/L; 10.0 mg/L and 50.0 mg/L of nTiO₂). We tested both mortality and three different sub-lethal responses: movement, net building and metabolism (i.e., biomarkers). We expected *H. exocellata* to respond significantly to nTiO₂ exposure, at least at the highest concentration.

Material and methods

Synthesis and characterisation of TiO₂ nanoparticles

Titanium dioxide nanoparticles (nTiO₂) were synthesised by sol-gel hydrothermal method (using SIGMA Aldrich® reagents) with Titanium (IV) butoxide (97%), 1-butanol (99.9%), pluronic P123 and nitric acid (69%) following the methodology described by Pol et al. (2016), which allowed us to obtain highly porous nanoparticles. We dissolved 7 ml of Titanium (IV) butoxide (97%) solution in 40 ml of 1-butanol. This mixture was added dropwise to 60 ml of distilled water (under a pH = 2 condition; previously adjusted with nitric acid 69%). The resulting solution was kept under agitation for 20 hours at room temperature. A hydrothermal treatment (80°C during two hours) was carried out to obtain a suspended solution. Next, 2.5 g of Pluronic P123 triblock copolymer was dissolved in 40 ml of distilled water and was added dropwise to the Titanium (IV) butoxide mixture that was previously cooled down to 45°C. This mixture was kept under agitation for two hours for a second hydrothermal treatment at 80°C during 20 hours. The resulting dust was recovered by evaporation at 60°C. Finally, the TiO₂ nanoparticles (nTiO₂) were obtained by calcification in an oven at 450°C during 4 hours (process in which the Pluronic P123 was removed). The resulting TiO₂ nanoparticles were characterised by High-Resolution Transmission Electron Microscopy (HRTEM, Jeol-JEM 2011, at 200 kV and with EDX detector) and by Scanning Electron Microscope (SEM, Merlin Zeis, at 2 kV) (Figure 1), which showed a mean particle diameter of 10 ± 2 nm. Samples for HRTEM and SEM were prepared by dispersing a 5 mg of TiO₂ powder in 300 µL of ethanol (96%) followed by sonication. A holey carbon grid was then soaked into the suspension and allowed to dry in air. The size distribution of TiO₂ nanoparticles (Figure S1) was determined via manual analysis of enlarged micrographs obtained by TEM. More than 100 particles were manually counted using ImageJ software in order to obtain the size distributions and the mean diameter. Selected Area Electron Diffraction (SAED) was used to determine the conformation patterns of the crystalline planes of nTiO₂ nanoparticles. On the other hand, Z potential analysis and Dynamic Light Scattering (DLS) (ZetaSizer Nano ZS,

Malvern Instruments) were performed to assess the formation of aggregates and nanoparticles' size in our experimental medium (river water), respectively.

Collection and acclimation of *Hydropsyche exocellata* Dufour, 1841

Individuals of *Hydropsyche exocellata* (4th instar larvae) were collected from a good water quality section of the Ter River (Les Masies de Voltregà, NE Catalonia, Spain) by carefully removing them from river cobbles using forceps. Then, they were transported to the laboratory in plastic bottles containing 5 litres of river water. Before performing the experiments, the *H. exocellata* individuals were acclimated to laboratory conditions for 10 days. Field conditions were recreated in the laboratory by using plastic trays with 4 litres of river water (resulting on approximate water depth of 15-20 centimetres, enough to fully cover the artificial habitat) that was pumped (using EHEIM® compact 300 pumps) to generate stable flow conditions (Figure 2). We used the artificial habitat created by Sala et al. (2016), which basically consisted of polystyrene blocks with stick sand that were separated from the pump by a net to prevent the individuals from being sucked. Several rows of toothpicks were inserted into the polystyrene and sewing thread was looped down each row like a fence. Toothpicks were jabbed in the sand at a uniform separation and were joined with sewing thread. *H. exocellata* successfully built nets around these artificial structures, which were easy to photograph. During the acclimation period, larvae were fed with algae collected from the river cobbles (once a week during acclimation and once a week during experimental period too).

nTiO₂ solutions and experimental design

A stock solution of 700 mg/L of TiO₂ nanoparticles was prepared in 1 litre of distilled water, and kept under agitation to maintain the TiO₂ nanoparticles suspended. All test solutions were prepared from the same stock (kept under agitation), by conducting dilutions in river water to achieve different test concentrations. Larvae were exposed to six different treatments, which included the control and five concentrations of TiO₂ nanoparticles (0.5 mg/L; 1.0 mg/L; 5.0 mg/L; 10.0 mg/L and 50.0 mg/L (Zhu and Cheng 2010)). Preliminary tests showed no effect of nanoparticles at concentrations under mg/L range. The TiO₂ nanoparticles were added only once throughout the experiment. We hypothesised that the initial concentration of nanoparticles and the manner in which nanoparticles entered to the artificial habitat (i.e., by a single higher-concentration or 'pulse'), would ultimately lead to differences in the distribution of

nanoparticles over the artificial habitat. We also hypothesised that the re-circulation of water in our artificial habitat could alter the environmental fate of the nanoparticles after being pumped. We assumed nanoparticles were recirculated during the first steps of the experiment; subsequently we expect the nanoparticles to form aggregates, interact with organic matter (mainly algae) and/or deposited over the sediment. As explained above, nanoparticles' size and stability in experimental medium was determined by Z potential and DLS at different exposure times and three different concentrations (0.5 mg/L, 5.0 mg/L and 50.0 mg/L).

Two independent experiments were carried out under similar experimental conditions. The trays were kept in an environmental chamber at a constant temperature of 14 °C and under dark conditions. Dark conditions were chosen because *Hydropsyche* naturally hide under the river cobbles, where light penetration is low, and they tend to be more active in the dark (Gerhardt 1996; Macedo-Sousa et al. 2008). Water was pumped constantly to maintain optimal levels of dissolved oxygen.

The first experiment was focused on monitoring mortality and abdominal contractions of the exposed *H. exocellata* larvae during a 14-day period. For this experiment, 10 larvae were added per tray, but after acclimation not all of them survived. Thus, the number of individuals that were exposed to treatment (using one tray per treatment) was the number of individuals after acclimation, which is provided in Table 1. Mortality was calculated in terms of percentage of death individuals after the 7- and 14-day period divided by the number of individuals when nanoparticles were added. The second experiment was focused on exploring changes in biomarker responses and net symmetry analyses in caddisfly larvae exposure to the same nTiO₂ concentrations as in the first experiment but shorter duration (i.e., seven days). Given that mortality was relatively high after acclimation in the first experiment, more individuals were used in the second experiment to assure that 10 living individuals per treatment could be used for biomarker analysis.

Abdominal contractions

Hydropsyche larvae have a very distinctive movement, contracting their abdomen rhythmically from side to side to increase oxygen uptake through the gills (Sala et al. 2016). To register these movements, individuals were placed on a petri dish with river water where the number of abdominal contractions (AC) was registered during 10

seconds. After the test, individuals were put back into the experiment tray. AC from four individuals per treatment were monitored twice a week during the experiment.

Net symmetry

The nets built by *H. exocellata* to capture food particles were photographed under light conditions before exposure to nTiO₂ (t = 0 days) and at the end of the experiment (t = 14 days) (Figure 3). Nets were photographed using a Nikon 7100 camera, fitted with a Sigma 17 70 f1:2.8 lens and a Sigma EM-140 DG flash. Pictures were taken always at the same distance. The images were then input to Matlab (MathWorks Inc.) for analysis following the same procedure explained in Sala et al. (2016) by using the energy distribution of 2D Discrete Cosine Transform (DCT) (Makhoul 1980) for each image. This analysis provided an entropy value as an indicator of the net structure. High entropy values were related with highly disorganised nets, whereas the low entropy values were related with high symmetry within the nets. The final entropy value was normalised using the number of nets built by *H. exocellata* per treatment divided by the number of individuals alive per treatment at the end of the experiment.

Biomarkers

For biomarker analysis, 10 individuals (i.e., whole body) of *H. exocellata* were collected per treatment (total n = 60) and immediately frozen in liquid nitrogen. Four biomarkers were analysed including one of cellular structure corruption lipid peroxidation (LPO), one antioxidant enzyme catalase (CAT), one indicator of general toxicity lactate dehydrogenase (LDH) and a phase II metabolic conjugate enzyme, glutathione S transferase (GST). Whole body (without the head) of *H. exocellata* was homogenised (in pools of two) in ice cold 0.1M phosphate buffer with 150mM KCl and 0.1mM ethylenediamine-tetraacetic acid, disodium, salt, dehydrate (EDTA) in a 1:8 (weight/volume) proportion. The homogenates were centrifuged at 10.000xg, 4°C during 20 minutes, and the supernatant was collected, aliquoted and stored at -80°C for biomarker determination.

Lipid peroxidation (LPO) was determined according to Esterbauer, Schaur and Zollner (1990) by measuring malondialdehyde (MDA), which is a well-known product of lipid peroxidation. The MDA assay is based on the reaction of the chromogenic reagent N-methyl-2-phenylindole (NMPI) with MDA at 45°C under acidic conditions, giving rise to a chromophore with absorbance at 586nm (Esterbauer et al. 1990). The

results are shown as nmol MDA equiv./ g ww (tissue wet weight). Catalase (CAT) activity was measured by monitoring the decrease in absorbance at 240nm due to H₂O₂ (52.5mM) consumption according to Aebi (1974). Results are shown as μ mol/min/mg of protein. Lactate dehydrogenase (LDH) activity was determined according to (Diamantino, Almeida, Soares, and Guilhermino 2001) by monitoring the absorbance decrease at 340 nm due to NADH (0.18mM) oxidation in the presence of Pyruvate (1.18mM). Results expressed as nmol/min/mg of protein. Glutathione S transferase (GST) activity towards 1-Cl-2,4-dinitrobenzene (CDNB) (1mM) in the presence of glutathione in its reduced form GSH (1mM) was measured as described by Habig et al. (1974). Results were normalised by tissue total protein content and expressed as nmol/min/mg of protein. Proteins were determined according to Bradford Method (Bradford 1976), using Bovine serum albumin (BSA) as standard. Except for catalase activity, where cuvette assay was used (Life Science UV/Vis Spectrophotometer DU® 730, Beckman Coulter – Fullerton, CA, USA), all the bioassays were performed in microplate (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instruments – Vermont, USA).

Statistical analyses

All statistical analyses were performed in R (R Core Team 2015). Firstly, we checked normality of all analysed variables using the Shapiro-Wilk normality test (shapiro.test function). Those variables that did not follow a normal distribution were transformed using the square root transformation. In order to assess the interaction between the time exposure to nTiO₂ and the number of AC, we performed a Two-Way ANOVA test. Number of AC, net symmetry and biomarker responses were analysed, respectively, by one-way analysis of variance (ANOVA), followed by Dunnett multiple comparison test to compare nTiO₂ toxicant effects with control treatment. Differences in mortality across time (7 days of exposure vs. 14 days of exposure) were analysed using non-parametric Kruskal-Wallis and X² test. Significant differences were established at $p < 0.05$.

Results

TiO₂ nanoparticles characterisation

The images obtained by HRTEM (Figure 1a) allowed us to measure the particle size. The diameter of TiO₂ nanoparticles' population used in this experiment ranged between

5 and 19 nm, with a mean diameter of 10 ± 2 nm. SAED pattern showed that, taking into account the conformation patterns of the crystalline planes, the main structure of the nTiO₂ was the anatase (JCPDS n° 84-1286). On the other hand, the morphology of the TiO₂ nanoparticles was characterised by SEM. In the micrographs obtained by SEM (Figure 1b) it is possible to observe the porosity of the synthesised nanoparticles. Z potential was measured to monitor the stability of the nanoparticles in three of our artificial habitats (Table S1) at different times of the experiment. Low values of Z potential indicated that the nanoparticles are electrically unstable in river water and hence tended to form aggregates in the experimental medium.

Mortality and behaviour

Throughout the 2 weeks of the experiment, mortality ranged from 0 to 22% and from 14 to 56% after 7 and 14 days of exposure, respectively (Table 1). No significant differences in mortality could be observed among the different exposure times (Kruskal Wallis, $X^2_5 = 5.7$; $X^2_5 = 3.9$). Mortality was generally high, including the control and the treatments with low nanoparticle concentrations (i.e., 0.5 and 1 mg/L). Nonetheless, the highest mortality (56%) was registered in the 50.0 mg/L treatment two weeks after pulse exposure.

The frequency of abdominal contractions (AC) increased significantly in a time and dose dependent manner (two-way ANOVA: $F = 4.8$, $p < 0.01$). Across all treatments, AC frequency tended to increase but was only significant at the highest nanoparticles concentration and time post exposure (50.0 mg/L, 336 h) ($p < 0.001$) (Figure 4). Overall, one-way ANOVA results showed statistical differences in net symmetry ($F = 7.67$, $p = 0.013$) two weeks after pulse exposure to nTiO₂ in all treatments (including the control) in comparison to the initial experimental conditions. However, when net symmetry was specifically compared between the control and the different treatments by Dunnett's multiple comparison test, no significant differences were found ($p > 0.05$).

Biomarker responses

Two of the four studied biochemical markers were significantly affected by nTiO₂ exposure (Figure 5). LPO levels increased in a dose dependent manner, being significantly higher than the control treatment at 50.0 mg/L (one-way ANOVA: $p = 0.019$). On the contrary, CAT activity significantly decreased in all treatments except at

50.0 mg/L (one-way ANOVA: $F = 3.01$, $p = 0.033$) (Figure 5). GST and LDH, were unaffected by nTiO₂ exposure.

Discussion

According to our results, pulse exposure to nTiO₂ did not have significant lethal effects on *H. exocellata*. In fact, mortality was relatively high across treatments, suggesting that acclimation to the artificial environment was challenging for some individuals. However, we observed some stress signals one week following exposure, which were characterised by changes in behaviour and prooxidant/antioxidant equilibrium.

The analysis of the Z potential of the nanoparticles in the exposure medium suggested certain stability of the nTiO₂ aggregates. Thus, the number of dissolved nanoparticles available in the water column could have been reduced during the experiment, leading to lower levels of active nanoparticles distributed over the experimental trays. Unfortunately, the size of the aggregates could not be determined by DLS analyses, but nTiO₂ are already known to form aggregates in various natural aqueous matrices, including river water (Keller et al. 2009). Thus, the interaction with suspended organic matter in the river water flowing through the artificial habitat could have led to coating and stabilization of nanoparticles (Baker et al. 2016). After initial pulse, nTiO₂ could have aggregated with the medium (river water, as quantified by Z potential), with the algae that are used to feed the *H. exocellata*, on the inner walls of the plastic trays, on the polystyrene blocks of the experimental setup, with humic substances, etc. This agglomeration modifies the size and charge of the nanoparticles and influences their toxicity and uptake. The continuous ingestion of the coated algae is the strongest hypothesis that supports the uptake of nTiO₂ by *H. exocellata* larvae. This has already been observed in daphnids that have ingested contaminated algae coated by nTiO₂ (Fouqueray et al. 2012). In that case, mortality was enhanced in neonates and minor effects over mortality, growth and reproduction was observed in adult individuals under continuous illumination conditions. Jackson et al. (2012) observed significant mortality among amphipods fed on algae contaminated with CdSe/ZnS quantum dots at a concentration equivalent to 3.6 mg/L of Cd of culture medium. Toxicity have also been observed in earthworms fed on food contaminated with CdS quantum dots at concentrations equivalent to 10 µg/L of Cd (Buffet et al. 2014); the induction of oxidative stress enzymes was observed too. As well as, an increase in caspase activity

and an alteration of body undulations was registered which support our obtained sub-lethal results. The effects of nanoparticles ingest *via* trophic chains has also been studied by Cedervall et al. (2012) which used a three-species trophic chain (algae – daphnids – fish) and found that polystyrene nanoparticles (at 0.01% w/v in algal culture medium) induced significant behavioural alteration and metabolic perturbations in *Danio rerio* Hamilton-Buchanan, 1822. These studies support that the continuous feed of coated algae (Resh 1992) could enhance their toxicity *via* the trophic route in *H. exocellata* larvae, which could explain our obtained results over net symmetry, behaviour and biomarker levels. Since, authors observed that the ingestion of nanoparticles induces greater toxicity than does direct exposure (Buffet et al. 2013; Fouqueray et al. 2013; Jackson et al. 2012).

The majority of ecotoxicological studies focusing on nTiO₂ and other metallic nanoparticles have used *D. magna* as test organism (Lovern and Klaper 2006; Adams et al. 2006; Hund-Rinke and Simon 2006; Warheit et al. 2007; Zhu and Cheng 2010). In this species, dose dependent increase of mortality correlated with increase of exposure time (Strigul et al. 2009; Zhu and Cheng 2010, Kim, Klaine, Cho, Kim, and Kim 2010), though with some differences. For example, Zhu and Cheng (2010) observed over 80% of mortality at concentrations higher than 5.0 mg/L of nTiO₂ after 48 hours of exposure, reaching to 100% at 50.0 mg/L when time of exposure was extended to 72 hours; whereas, Kim et al. (2010) were not able to calculate the LC50 in the acute bioassay (48 hours) at nTiO₂ concentrations of 5.0 and 10.0 mg/L, but a chronic bioassay (21 days) showed mortality levels of 70 and 80% respectively for 5.0 and 10.0 mg/L of nTiO₂. Thus, in summary, previous studies on *D. magna* suggest that a concentration of 5.0 mg/L should trigger some kind of toxic response on this organism, while a concentration of 50.0 mg/L should have drastic effects. In our study, most extreme scenario of nTiO₂ exposure (50.0 mg/L) led to the highest mortality at both time periods, but then again, did not differ significantly from control treatments provided that in all treatments including controls about 10–20% mortality occurred after the acclimation phase. Even though mortality was not significantly affected by treatment, we did observe significant changes in AC and biomarker activity.

Net symmetry analysis has been used in other studies to evaluate potential effects of pollutants on *Hydropsyche* species (Becker 1987; Petersen and Petersen 1983; Tessier et al. 2000; Sala et al. 2016; Wendt-Rasch, Vought, and Woin 1998). Changes in the structure of the otherwise symmetrical capture-nets can be regarded as a sub-

lethal behaviour response to stress and might be of potential use in a biomonitoring program (Wendt-Rasch et al. 1998). Various toxic substances have been reported to affect the net-spinning behaviour of the larvae of the trichopteran genus *Hydropsyche*. Exposure to different pollutants like DDT (Décamps, Besch, and Vobis 1973), fenethcarb (Besch, Schreiber, and Herbst 1977), copper (Besch et al. 1979), pulp mill effluent and trichloroguaiacol (Petersen and Petersen 1984) have been reported to increase the frequency of structural deformities in hydropsychid larvae's capture-nets under laboratory conditions. An increased frequency of anomalies has also been found in nets collected from streams polluted by heavy metals (Petersen and Petersen 1983). In our study, net symmetry was not significantly affected by nTiO₂ exposure. This could be due to an absence of effect of nTiO₂ on this behaviour at the concentrations tested. However, it could also be related with a methodological issue. Our method has only been tested once to detect salt toxicity on streams (Sala et al. 2016). Previous studies analysing *Hydropsyche* nets' symmetry carefully retrieved the nets and measured them under the microscope (Becker 1987; Tessier et al. 2000; Wendt-Rasch et al. 1998). Since that was a very time-consuming process, our method could be a promising alternative for routinely measuring this sub-lethal endpoint. However, a comparison of the method used here with previous methods for analysing net symmetry seems necessary.

Additionally, we assessed changes in locomotion as a behavioural response to pollution. The larvae of *H. exocellata* have a very distinctive movement of its abdomen (i.e., abdominal undulatory movements) that are related to the increment in the surface area with water, that allows the caddisfly to obtain higher quantities of dissolved oxygen through the gills (Sala et al. 2016). van der Geest (2007) observed that the average time spent on ventilation of the caddisfly *Hydropsyche angustipennis* Pictet, 1834 increased significantly from 3% under optimal oxygen saturation conditions (i.e., 100%), to 25% when oxygen concentration was reduced to 30%. In our study, the exposure to nTiO₂ caused a significant change in abdominal contractions (AC), especially at 50.0 mg/L. This behaviour response could be explained by the bioaccumulation of nTiO₂ in the larvae's gills, since nanoparticles have a high specific surface area reactivity (Lovern and Klaper 2006) that allows them to react and accumulate within animal tissues (Carlson et al. 2008; Ispas et al. 2009; Mironava et al. 2010). In marine invertebrates, specifically in the bivalve mollusc *Mytilus spp.*, *in vivo* exposure to nanoparticles indicated that, due to the physiological mechanisms involved

in the feeding process, nanoparticle's agglomerates/aggregates were taken up by their gills and were directed to the digestive gland, where intra-cellular uptake of nanosized materials induced oxidative stress (Canesi et al. 2012). Thus, nTiO₂ could pass through the gills towards the body cells (Carlson et al. 2008; Ispas et al. 2009; Mironava et al. 2010). In a similar way, the accumulation of nanoparticles in the larvae's gills could lead to an increase in undulatory movements to facilitate ventilation and oxygen uptake. Several studies have also reported an increase in AC of *Hydropsyche* as response to water pollution (Gerhardt 1996; Sala et al. 2016), whereas others did not find a clear evidence of AC movements with toxic stress (Macedo-Sousa et al. 2008; Sala et al. 2016). As suggested previously by Sala et al. (2016), this behavioural response deserves further study.

Biomarkers have been shown to be more sensitive indicators than traditional toxicity testing of how nanoparticles may cause alterations in the organism physiology (Klaper et al. 2009). Additionally, nTiO₂ is known to produce reactive oxygen species (ROS) (Colvin 2003; Klaine et al. 2008; Xiong, Fang, Yu, Sima, and Zhu 2011) and cause oxidative stress that can lead to cell and tissue damage. In our study, the presence of oxidative tissue damage caused by 50.0 mg/L of nTiO₂ was confirmed by increment of LPO levels. As mentioned previously, the majority of data available concerning ecotoxicity of nTiO₂ in freshwater invertebrate species are of crustacean species, mainly *D. magna*. Nonetheless, some ecological studies showed that nTiO₂ exposure caused oxidative damage-mediated effects in other aquatic species (Federici, Shaw, and Handy 2007; Jemec, Drobne, Remskar, Sepcic, and Tisler 2008; Klaper et al. 2009). For example, the exposure of rainbow trout to nTiO₂ caused lipid peroxidation, in the gill, intestine and brain tissue (Federici et al. 2007). Also, evidence of LPO analysis as biomarker for oxidative tissue damage derived by poor water quality and toxic stress in *H. exocellata* has already been reported (Barata et al. 2005, Damásio et al. 2011, Sala et al. 2016). Organisms responses against ROS, in order to avoid cellular damage, usually involve the increase of antioxidant enzyme activities such as superoxide dismutase enzyme (SOD) that convert the superoxide anion radical O₂⁻ to hydrogen peroxide (H₂O₂) and catalase or glutathione peroxidase, as a second line of defence, that further reduces H₂O₂ to water (Halliwell and Gutteridge 1999). Indeed, studies in nTiO₂ exposed *D. magna* and *Mytilus galloprovincialis*, Lamarck, 1819 showed that CAT activity increased with nTiO₂ concentration (Kim et al. 2010; Canesi et al. 2012). Our results obtained for CAT activity suggest the opposite, i.e., CAT antioxidant enzyme

activity was inhibited when nTiO₂ doses increase. This inhibition may be related to the production of excess superoxide anion, resulting from the failure of superoxide dismutase enzyme to convert it into hydrogen peroxide (Halliwell and Gutteridge 1999; Kono and Fridovich 1982), hence resulting in a flux of O₂⁻ radicals which are known to inhibit CAT activities, thus suggesting that caddisfly larvae, when exposed to higher concentrations of nTiO₂, could be more susceptible to suffer oxidative stress. Indeed, the inhibition trend of CAT activity was coupled with that observed for tissue damage (LPO) increase. The fact that CAT activity was not significantly lower in the 50 mg/L treatment than in the control treatment suggests that it could be recovering due to increase of enzyme synthesis as a feedback response to activity inhibition (Chan et al. 2006). However, CAT activity recovery was not sufficient to decrease oxidative damage. The remaining two biomarkers (GST and LDH) were not affected by nTiO₂, which indicate that mechanisms of phase II biotransformation and cell anaerobic metabolism of *H. exocellata* were not affected by nTiO₂ toxicity.

Conclusion

In this study is shown the effect of nTiO₂ over an aquatic insect species, which represents an intermediary trophic level between primary producers and fish species in riverine ecosystems. After a pulse nTiO₂ exposure to *H. exocellata* larvae, we demonstrate that nTiO₂ at 50.0 mg/L concentration can have a toxic effect on the caddisfly *H. exocellata* larvae by altering behaviour and triggering subcellular responses related with oxidative stress. Even at this very high concentration, the response of *H. exocellata* was rather weak, suggesting that nTiO₂ pollution is not posing a high risk to this species. However, since *H. exocellata* is considered as a pollution tolerant species (Bonada et al. 2004), our results suggest that high nTiO₂ concentrations (50 mg/L) could have significant effects on other aquatic invertebrates. It is also important to notice that these concentrations of nTiO₂, which are harmful to *H. exocellata*, are very high, around 5–6 orders of magnitude from those reported in surface waters for different regions of Europe by Gottschalk et al. (2009). But, as mentioned by previous studies, nTiO₂ is rapidly aggregated in river water and interact with organic matter and algae which represents an important route of entry *via* ingest which is enhancing its toxicity at our nTiO₂ used concentrations. Thus, the concentrations accumulated in the bodies of river organisms like *H. exocellata* could be significantly higher than those found in the water. In a similar way, Tszedel, Markowski, Majecki, Błońska, and Zieliński (2015)

reported that the metal concentration in the body of *H. angustipennis* was much higher than the water concentration. Moreover, it has been estimated that the alarming increasing use of ENM, including nTiO₂, (Barrena et al. 2009; Gottschalk et al. 2009; Moore 2006), will dramatically increase the environmental concentrations of these contaminants in the future. Before maximum allowed concentrations of nTiO₂ in river waters can be proposed, more studies are needed including further physico-chemical characterization and different exposure conditions, model organisms and biological responses.

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Table 1. Percentage of dead individuals with respect to the initial number of individuals (after acclimation) 7 and 14 days after treatment. Mortality (%) = (n° death individuals / initial number of individuals) * 100

Figure 1. Images of TiO₂ anatase nanoparticles used for this experiment obtained by sol-gel hydrothermal method. The A micrographs were taken by HRTEM (Jeol-JEM 2011, at 200kV and EDX detector) where it is observed the morphology of this nanoparticle population that was clearly homogeneous with mean diameter of 10 ± 2 nm. The B micrographs show the synthesised nanoparticle taken by SEM where it can be seen porosity of this nanoparticle.

Figure 2. Image of the experimental trays, adapted from Sala et al. (2016). Water was constantly pumped (the pump can be seen in the upper right corner of the tray) to create stable flow conditions.

Figure 3. Images of the nets built by larvae of *Hydropsyche exocellata* Dufour, 1841 larvae taken by Nikon 7100 camera, fitted with Sigma 17 70 f1:2.8 lens and a Sigma EM-140 DG flash. Image A and B show a net built by the larvae, respectively, before and after adding 50 mg/L of TiO₂ nanoparticles. These two images show the clear visual differences between these two nets, which their symmetry were found to be significantly different after applying nTiO₂ (p = 0.013, ANOVA test).

Figure 4. Plots of the number of abdominal contractions (AC) of larvae of *Hydropsyche exocellata* Dufour, 1841 larvae across dose/time of exposure to nTiO₂. Significant responses of all treatments when compared to control are indicated by *. Dunnett's post hoc multiple comparison test showed that the control (p < 0.001, Dunnet test) treatment was significantly different when compared the number of AC with the 50.0 mg/L treatment in *H. exocellata* larvae.

Figure 5. Biomarker responses of whole body tissue of *Hydropsyche exocellata* Dufour, 1841 larvae exposed to increasing doses of nTiO₂ for 7 days: (a) levels of lipid peroxidation (LPO) (nmol MDA equiv./g ww); (b) catalase (CAT) activity (µmol/min/mg); (c) glutathione S transferase (GST) activity (nmol/min/mg); (d) lactate dehydrogenase (LDH) activity (nmol/min/mg). * p < 0.05, following ANOVA and

Dunnett's post hoc multiple comparison test. Bars indicate the standard deviation (n =10 individuals per treatment).

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Figure 1

a)

b)

Figure 2: Image of the experimental trays, adapted from Sala et al., (2016). Water was constantly pumped (the pump can be seen in the upper right corner of the tray) to create stable flow conditions.

338x190mm (96 x 96 DPI)

Figure 3: Images of the nets built by *H. exocellata* larvae taken by Nikon 7100 camera, fitted with Sigma 17 70 f1:2.8 lens and a Sigma EM-140 DG flash. Image A and B show a net built by the *H. exocellata* larvae, respectively, before and after adding 50 mg/L of TiO₂ nanoparticles. These two images show the clear visual differences between these two nets, which their symmetry were found to be significantly different after applying nTiO₂ (p=0.013, ANOVA test).

338x190mm (96 x 96 DPI)

Figure 4: Plots of the number of *H. exocellata* larvae abdominal contractions (AC) across dose/time of exposure to $n\text{TiO}_2$. Significant responses of all treatments when compared to control are indicated by *. Dunnett's post hoc multiple comparison test showed that the control ($p < 0.001$, Dunnett test) treatment was significantly different when compared the number of AC with the 50.0 mg/L treatment in *H. exocellata* larvae.

338x190mm (96 x 96 DPI)

Figure 5: Biomarker responses of whole body tissue of *H. exocellata* larvae exposed to increasing doses of nTiO₂ for 7 days: (A) levels of lipid peroxidation (LPO) (nmol MDA equiv./g ww); (B) catalase (CAT) activity (μmol/min/mg); (C) glutathione S transferase (GST) activity (nmol/min/mg); (D) lactate dehydrogenase (LDH) activity (nmol/min/mg). * p<0.05, following ANOVA and Dunnett's post hoc multiple comparison test.

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Treatment	% Mortality	
	7 days	14 days
Control	11	22
0.5 mg/L	0	33
1 mg/L	20	30
5 mg/L	14	14
10 mg/L	14	29
50 mg/L	22	56

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Figure S1. The size distribution of TiO₂ nanoparticles

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Table S1. Z Potential values of the TiO₂ samples for the artificial habitat using different concentrations stock at different times.

Concentration of TiO ₂ (mg/L)	Time (days)	Z Potential (mV)
50	0	-20.5
	2	-18.3
	6	-20.5
	9	-11.8
	15	-17.3
5	0	-13.9
	2	-20.3
	6	-15.7
	9	-15.0
	15	-17.7
0.5	0	-18.4
	2	-16.8
	6	-11.5
	9	-14.1
	15	-18.3