

Minimal levels of ultraviolet light enhance the toxicity of TiO₂ nanoparticles to two representative organisms of aquatic systems

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Abstract A number of studies have been published concerning the potential ecotoxicological risks of titanium dioxide nanoparticles (nano-TiO₂), but the results still remain inconclusive. The characteristics of the diverse types of nano-TiO₂ must be considered in order to establish experimental models to study their toxicity. TiO₂ has important photocatalytic properties, and its photoactivation occurs in the ultraviolet (UV) range. The aim of this study was to investigate the toxicity of nano-TiO₂ to indicator organisms of freshwater and saline aquatic systems, under different illumination conditions (visible light, with or without UV light). *Daphnia similis* and *Artemia salina* were

co-exposed to a sublethal dose of UV light and different concentrations of nano-TiO₂ in the form of anatase (TA) or an anatase/rutile mixture (TM). Both products were considered practically non-toxic under visible light to *D. similis* and *A. salina* (EC_{50,48h} > 100 mg/L). Exposure to nano-TiO₂ under visible and UV light enhanced the toxicity of both products. In the case of *D. similis*, TM was more toxic than TA, showing values of EC_{50,48h} = 60.16 and 750.55 mg/L, respectively. *A. salina* was more sensitive than *D. similis*, with EC_{50,48h} = 4 mg/L for both products. Measurements were made of the growth rates of exposed organisms, together with biomarkers of oxidative stress and metabolism. The results showed that the effects of nano-TiO₂ depended on the organism, exposure time, crystal phase, and illumination conditions, and emphasized the need for a full characterization of nanoparticles and their behavior when studying nanotoxicity.

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Introduction

TiO₂ is a crystalline semiconductor that possesses the important property of being able to be photoactivated at wavelengths in the range of 300–338 nm (Gaya and Abdullah 2008). The naturally occurring crystalline

phases of TiO₂ are anatase, rutile, and brookite, of which anatase and rutile are the most common. The anatase form shows the highest photocatalytic activity, but there is evidence of synergism between the crystal phases, and that an anatase/rutile blend is more photoactive, compared to the pure phases.

Since it is already widely used, and also shows promise in new emerging applications, nano-TiO₂ has been the subject of a number of ecotoxicological investigations. However, questions have been raised concerning both the suitability of existing nanoecotoxicological protocols and the need for standardization (Handy et al. 2012).

The photoactivation of nano-TiO₂ generates reactive oxygen species (ROS) able to degrade organic and inorganic compounds (Chatterjee and Dasgupta 2005; Fujishima and Zhang 2006). The ROS production has also been proposed to be the principal cause of the material toxicity toward different organisms. An imbalance between the production of ROS and the antioxidant systems of the organism can lead to a condition known as oxidative stress (Hwang and Kim 2007). Ma et al. (2012a) reported that the immobilization of *D. magna* exposed to nano-TiO₂ was related to the production of ROS. Another mechanism of toxicity could be by physical action; it has been suggested that the toxicity of nano-TiO₂ to *Daphnia* may result from reduced food consumption due to the ingestion of agglomerates of the nanomaterial (Seitz et al. 2013). Use of nuclear microscopy indicated that Ti was only located in the digestive tract of *Daphnia* exposed to nano-TiO₂ (Keller et al. 2010). However, in work using confocal microscopy, Li (2011) found that Ti was present in the digestive tract, tissues, brood chamber, and appendages of *Ceriodaphnia dubia*. The authors suggested that nano-TiO₂ was unable to penetrate the gut wall, and proposed an exposure route involving direct contact, ingestion, and internal contact with tissues and embryos. In another study, Braydich-Stolle et al. (2009) reported that pure anatase induced rupture of the keratinocyte membrane, leading to necrosis.

A wide range of EC50 values (the concentration that causes toxic effects in 50 % of the exposed population) have been reported for microcrustaceans exposed to nano-TiO₂ (Clemente et al. 2012). Most studies describe the substance as being non-toxic to *Daphnia* (Griffith et al. 2008; Heinlaan et al. 2008; Lee et al. 2009; Kim et al. 2010; Rozenkranz 2010),

although EC50_{48h} values of 26.6 mg/L (Wiench et al. 2009) and 42 mg/L (Li 2011) have also been reported. Extension of the exposure period from 48 to 72 h appeared to substantially increase the toxicity of the nano-TiO₂, with EC50_{72h} values of 1.30, 3.15, and 3.44 mg/L for anatase sizes of 15, 25, and 32 nm, respectively (Clément et al. 2013), and a value of 1.62 mg/L for P25 (Zhu et al. 2010).

There are few studies that have evaluated the toxicity of nano-TiO₂ in *Artemia*, an important indicator organism in inland saltwater ecosystems. Barelds (2010) exposed *Artemia nauplii* to concentrations up to 10 mg/L of nano-TiO₂ (20 nm), and were not able to establish an EC50_{24h}.

In bioassays employing aquatic organisms, the circadian cycle is usually established using fluorescent lamps. These mainly emit visible light, while under natural conditions the organisms are exposed to solar radiation [infrared, visible, and ultraviolet (UV) light]. The photocatalytic properties of nano-TiO₂ can increase its toxicity to aquatic organisms under natural conditions, and examples of this can be found in the literature (Ma et al. 2012b; Marcone et al. 2012; Clemente et al. 2013; Xiong et al. 2013).

The determination of concentrations that cause no adverse effect on physiological parameters in a longer exposure time is extremely relevant to propose maximum allowable limits for water bodies. The use of biomarkers to evaluate risk has the advantage of enabling early detection of potentially toxic exposure. Although some studies have not found any changes, others have described increases or decreases in the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and peroxidase, in aquatic organisms exposed to nano-TiO₂ (Finnegan et al. 2007; Hao et al. 2009; Scown et al. 2009; Kim et al. 2010). The effects of nano-TiO₂ have not been evaluated for other equally important enzymes such as the phosphatases, which are involved in a variety of transphosphorylation reactions and can be affected by metals and ROS (Östman and Böhmer 2001).

The objective of this study was to evaluate the acute and long-term toxicity of different formulations of nano-TiO₂ to microcrustaceans exposed under varying illumination conditions, by observing their mobility, biochemical analysis, and growth rate assessments. Ecotoxicological tests were conducted using organisms that were representative of the same trophic level

in different aquatic ecosystems: freshwater (*Daphnia similis*) and saltwater (*Artemia salina*).

Materials and methods

Characterization of the NPs and their stability in suspension

Evaluation of the nano-TiO₂ toxicity in microcrustaceans was performed using titanium (IV) oxide nanopowder (“TA”—Sigma Aldrich, 100 % anatase, primary particle size < 25 nm, 99.7 % purity) and Aeroxide P25 (“TM”—Degussa Evonik, 20 % rutile, 80 % anatase, primary particle size 25 nm, 50 m²/g, 99 % purity). These commercial products have been extensively studied, and their measured characteristics have been reported to be very close to the manufacturers’ specifications (Federici et al. 2007; Grassian et al. 2007; Griffith et al. 2008; Palaniappan and Pramod 2010).

Stock suspensions (1 g/L) of each culture media used (“[Test organisms and culture media](#)” section) were prepared by sonication for 10 min using a high frequency probe (CPX600 Ultrasonic Homogenizer, Cole Parmer, USA) operated at 120 W/L. Immediately after the sonication, suitable volumes were removed in order to prepare suspensions at concentrations of 1, 10, and 100 mg/L under the same bioassay conditions (dilution in the *Daphnia* or *Artemia* culture media).

The hydrodynamic size, surface charge (zeta potential, ZP), and polydispersion index (PdI) of the particles in the 100 mg/L suspensions were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 instrument (Malvern Instruments, UK). The colloidal stability of suspensions during 24 h was evaluated by means of optical spectra obtained in the wavelength range of 200–600 nm using a UV–Vis spectrophotometer (Model 1650PC, Shimadzu, Japan). For each suspension, the precipitation rate was calculated from the angular coefficient of the linear regression curve obtained using the logarithm of the absorbances at 325 nm.

Test organisms and culture media

The *D. similis* culture and exposure media were prepared using tap water that had been previously filtered for 48 h using a filter containing activated

carbon. The characteristics of the water used for the *Daphnia* were as follows: pH 7.9 ± 1 ; conductivity $133 \pm 1 \mu\text{S}/\text{cm}$; total hardness 2°dGH ; temperature $20 \pm 1^\circ \text{C}$; and dissolved oxygen (DO) $6 \pm 0.5 \text{ mg}/\text{L}$.

Nauplii of *A. salina* were obtained after 48-h incubation of commercial cysts of Artemia (Maramar[®], Brazil) in 3 % saline solution (Red Sea Salt[®]) prepared with distilled water. For the growth tests, saccharose (3 %) was added to the exposure to prolong the test, as it is used by the artemia as a source of energy (Dvorak et al. 2012; Toi et al. 2013). The characteristics of the water used for the *Artemia* were as follows: pH 8 ± 1 ; conductivity $40 \pm 1 \text{ mS}/\text{cm}$; temperature $20 \pm 1^\circ \text{C}$; and DO $6 \pm 0.5 \text{ mg}/\text{L}$.

The bioassays were performed using small glass Petri dishes (5-cm diameter) containing 10 mL of exposure medium. All the tests were performed in quadruplicate, with five individuals for each replicate ($n = 20$ per group).

Illumination conditions

In the laboratory, visible light was provided from standard 40 W fluorescent lamps (Phillips), and UV light exposure was provided using a reflector containing two lamps UVA-340 Q-Panel (40 W). Measurements of artificial UV radiation were performed using a spectroradiometer (Model USB 2000 + RAD, Ocean Optics, USA) and a radiometer (Model VLX-3W, Cole Parmer, USA) with different sensors for UVA ($365 \pm 2 \text{ nm}$), UVB ($313 \pm 2 \text{ nm}$), and UVC ($254 \pm 2 \text{ nm}$). The intensity of visible light in the laboratory was measured using a digital lux meter (Model LD-500, ICCEL, Brazil). The regions of the electromagnetic spectrum considered in spectroradiometer measurements were those adopted by the International Commission on Illumination (CIE 1999): visible light (400–700 nm), UVA (400–315 nm), UVB (315–280 nm), and UVC (280–200 nm).

The intensity of visible light in the laboratory was $250 \pm 79 \text{ lux}$. At the height at which the tests were conducted, no UV radiation was detected from the fluorescent lamps. No UVC radiation was detected during the assays. The spectrum of the UVA-340 lamps was from 300 to 610 nm, with an irradiance peak at 340 nm (Supplementary information 1). Attenuation of the UVA and UVB radiation, as a function of distance from the lamps, is illustrated in Supplementary information 2.

Acute toxicity test—ultraviolet radiation

To determine the ED₅₀ values (the dose required to affect 50 % of the exposed population) of UV exposure, the organisms were exposed to distances between 10 and 110 cm from the UV lamps, for a time interval of 48 h. The exposure was made in Petri dishes containing the organism respective culture media. A control group was kept in the same room, but without exposure to the UV radiation.

Acute toxicity test—nano-TiO₂

The toxicity of each nano-TiO₂ formulation to *Daphnia* and *Artemia* was evaluated during a 48 h period, using two illumination conditions: visible light (visible light groups) or UV and visible light (UV light groups). Neonates of *D. similis* were kept at a distance of 150 cm from the lamps, corresponding to a total UV dose of 0.046 mW/cm² (0.17 J/h/cm²), as measured using the spectroradiometer. The *Artemia* were kept at a distance of 65 cm from the lamps, corresponding to a total UV dose of 0.6 mW/cm² (2.3 J/h/cm²). These UV radiation doses were equivalent to 5 % of the UV ED_{50,48h} calculated for each species.

The exposure media were prepared as described in “[Characterization of the NPs and their stability in suspension](#)” section, by diluting the stock suspension in the appropriate culture medium for each test organism. The organisms were not fed during the tests, and the exposure media were totally renewed after 24 h because of the suspension instability.

Pre-tests were performed to establish the concentrations of nano-TiO₂ to be used in the final tests. Under visible light, the *Daphnia* were exposed to 0 (control), 100, and 1000 mg/L of both nano-TiO₂ formulations. Under UV light, the *Daphnia* were exposed to 0, 6.25, 12.5, 25, 50, and 100 mg/L of TM, and to 62.5, 125, 250, 500, and 1000 mg/L of TA. Under visible light, the *Artemia* were exposed to 0, 250, 500, and 1000 mg/L of TA, and to 166, 500, and 1000 mg/L of TM. Under UV light, the *Artemia* were exposed to 0, 5, 10, 20, 40, and 80 mg/L of TA, and to 1, 2, 5, 10, and 20 mg/L of TM.

At the end of the exposure time, the total of individuals showing mobility in each recipient was registered. The data were used to determine the EC_{50,24h} and EC_{50,48h} values.

Growth test

In order to identify the occurrence of any sublethal effects on the microcrustaceans exposed to nano-TiO₂, the growth rates of the *Daphnia* and *Artemia* exposed to TA and TM were evaluated using the same illumination conditions described for the acute toxicity tests (“[Acute toxicity test—nano-TiO₂](#)” section). *D. similis* neonates and nauplii of *A. salina* were exposed for 96 h to concentrations that were shown not to cause any immobility in pilot experiments. The *Daphnia* were exposed to 0 (control), 1, and 10 mg/L of TA and TM, under visible light or UV and visible light. The *Artemia* were exposed to 0 (control), 0.06, and 0.6 mg/L of TA and TM, under visible light or UV and visible light. The concentrations used were equivalent to around 1.5 and 15 % of the lowest nano-TiO₂ EC_{50,48h} found for each organism.

The bioassays were performed using uncovered 24-well polystyrene plates, with the organisms ($n = 10$ per group) kept individually in wells containing 2 mL of solution. The suspensions were totally renewed on a daily basis, and the organisms were fed once daily (after renewal of the exposure media) with dehydrated *Chlorella pyrenoidosa* (1 drop of 2 g/L suspension per well).

The organisms were photographed at the beginning of the test and after every 24 h, using an Optika 4083B3 camera coupled to a stereomicroscope (Optika, Italy). Measurement of the size of the organisms was performed using Optika View (v. 7.1.1.5) software that had been previously calibrated using a graduated slide. The growth rates were evaluated by calculating the angular coefficients of the linear regression curves for the growth of individual organisms after 96 h.

Biochemical analysis

The *D. similis* were exposed for 24 h to different concentrations of TA and TM, under the same illumination conditions described for the acute test. In addition to control groups, the following concentrations were tested: 7.5, 75, and 750 mg/L of TA, and 0.6, 6, and 60 mg/L of TM. For each formulation, the concentrations tested corresponded to 1, 10, and 100 % of the EC_{50,48h} under UV light. Around 100 adult organisms (at least three replicates) were exposed to the test conditions in Petri dishes (10-cm

diameter, 1-cm water column height) containing 50 mL of solution. At the end of the exposure period, the organisms were collected using a sieve (21.2-mm mesh), washed with distilled water, weighed, and stored at -80°C prior to the biochemical analyses.

The samples were homogenized in 50 mM phosphate buffer (pH 7), using a 1:10 mass/volume ratio, and centrifuged at $10,000\times g$ for 10 min at 4°C . The supernatant was used to determine the activities of GST (Keen et al. 1976), glutathione peroxidase (GPx; Sigma 2000), CAT (Aebi 1984), SOD (Ukeda et al. 1997), and acid phosphatase (AP; Prazeres et al. 2004). All measurements were made in triplicate.

The data obtained from the different biochemical assays were normalized according to the total protein contents of the samples, quantified using the Bradford method (Bradford 1976), using a standard curve with bovine serum albumin (BSA).

Statistical analysis

The 24- and 48-h ED₅₀ values (the dose required to affect 50 % of the exposed population) of UV exposure and the 24- and 48-h EC₅₀ (the concentration required to affect 50 % of the exposed population) of nano-TiO₂ exposure, together with the corresponding 95 % confidence intervals, were calculated using probit analysis (Statgraphics Plus v. 5.1 software). The EC₅₀ values were considered to be statistically different when there was no overlap of the 95 % confidence intervals. The growth rates and the biochemical data were compared using two-way ANOVA. The factors considered for each exposure were the illumination conditions (with and without UV), the concentrations of TA and TM, and the interaction between these parameters. The Holm-Sidak post-test was used to compare the groups, using a significance level of 5 %. The normality of the data was evaluated using the Shapiro–Wilk test. These analyses employed Sigma Plot (v. 11.0) software.

Results

Characterization of the NPs and their stability in suspension

The absorbances of the 1 mg/L suspensions were very close to those of the media without nano-TiO₂

(blanks), so it was not possible to correctly evaluate either this or lower concentrations. The nano-TiO₂ suspensions presented an absorbance peak at 325 nm, and this wavelength was used to determine the precipitation of the suspensions over the course of time (Fig. 1). The nano-TiO₂ suspensions showed substantial instability, and precipitation was greater at higher concentrations. The 100 mg/L suspension of TM precipitated at a rate that was between 1.2 and 2.5 times higher than that of TA, depending on the medium used (Supplementary information 3). The opposite was observed for 10 mg/L suspensions, where the precipitation rate of TA was between 1.3 and 2 times greater than that of TM.

The 100 mg/L suspensions of TM showed rapid reductions of absorbance for all three media evaluated, with values equivalent to around 24, 9, and 3 % of the initial absorbances after 3, 6, and 24 h, respectively. Reductions in the absorbances of the 10 mg/L suspension of TM were more gradual. The absorbance of the 10 mg/L suspensions diminished to 84, 63, and 30 % of the initial value after 3, 6, and 24 h, respectively.

The absorbance of the TA suspensions in the *Artemia* media diminished more abruptly, compared to the *Daphnia* media. In the first 3 h, the absorbance of the 100 mg/L suspensions of TA reduced to 10, 20, and 43 % of the initial value for the media containing *Artemia*, *Artemia* with saccharose, and *Daphnia*, respectively. After 24 h, 3, 7, and 24 %, respectively, of the initial absorbances were measured in these same media. The suspensions containing 10 mg/L of TA precipitated more progressively. After 3 h, the absorbances of the 10 mg/L suspensions of TA diminished to 34, 49, and 70 % of the initial values, for the media containing *Artemia*, *Artemia* with saccharose, and *Daphnia*, respectively. After 24 h, around 20 % of the initial absorbance was measured.

Due to the instrumental detection limit, the DLS measurements were only conducted for the 100 mg/L suspensions (Table 1). The intense formation of aggregates and rapid precipitation of nano-TiO₂ affected the quality of the readings, as evidenced by the high values of the PdI obtained for all the suspensions. Hence, although single particle population peaks at around 700 nm were detected for the suspensions of TA in the three media, Z-average values above 1 μm were obtained in almost all cases. The presence of ions in the media, pH values close to

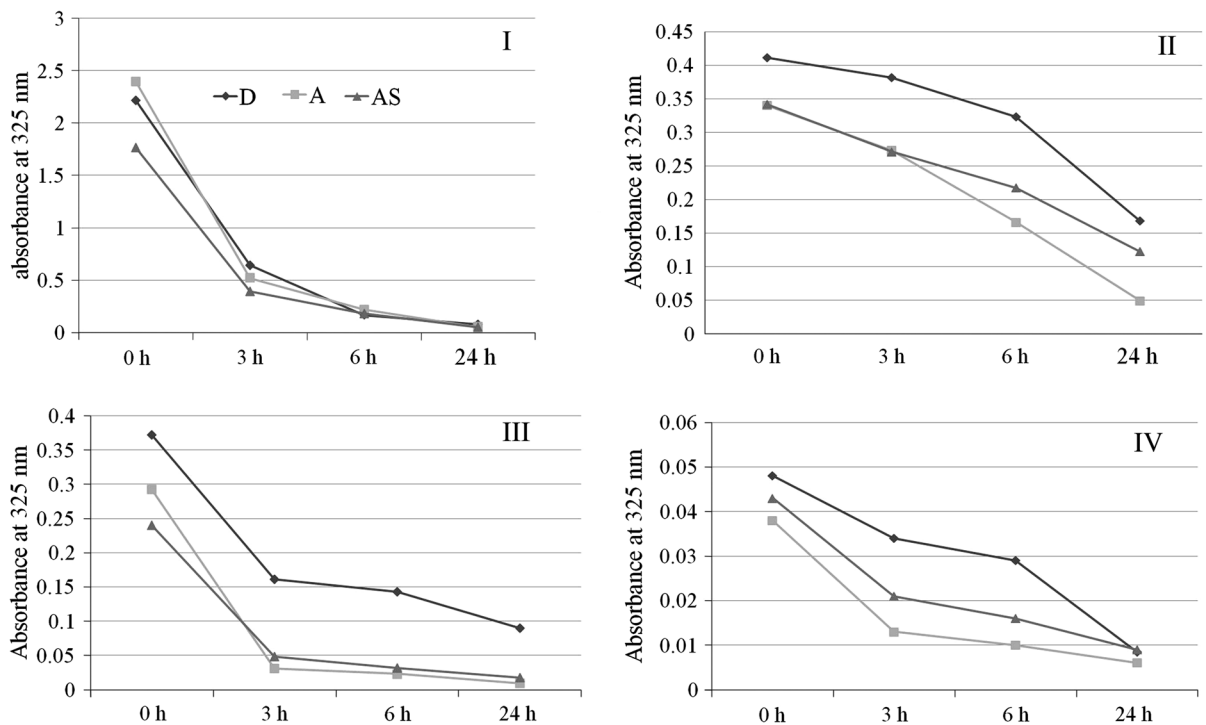


Fig. 1 Colloidal stability of the nano-TiO₂. Absorbances at 325 nm of the suspensions of nano-TiO₂ in the exposure media for *Daphnia* (D), *Artemia* (A), and *Artemia* with saccharose

(AS), according to time. **I** 100 mg/L TM; **II** 10 mg/L TM; **III** 100 mg/L TA; **IV** 10 mg/L TA

the pH_{Zpc} of nano-TiO₂, and low ZP values probably contributed to the instability of the suspensions. Intense aggregation and precipitation of nano-TiO₂ in the exposure media are in agreement with earlier findings (Ma et al. 2012b; Pagnout et al. 2012).

Acute toxicity tests

In all the acute toxicity tests, survival in the control groups exceeded 90 % after 48 h exposure, confirming that the experimental conditions were satisfactory (OECD 202, 1984). The distances of the UV lamps and the corresponding UV doses required to immobilize 50 % of the exposed *D. similis* are presented in Table 2.

The toxicity of the UV radiation (280–400 nm range of wavelength) to *A. salina* was tested to distances of up to 10 cm (a dose of 3.5 mW/cm² equivalent to 12.7 J/h/cm²), and no immobility was observed for exposure periods of up to 48 h. However, at a lamp distance of 10 cm, the rapid evaporation of water hindered the bioassays. Evaluation of the

toxicity of the nano-TiO₂ under UV radiation, therefore, employed a lamp distance of 65 cm, corresponding to 0.6 mW/cm² (2.3 J/h/cm²), which was 20 times lower than the ED₅₀_{48h} (13 mW/cm², equivalent to 47 J/h/cm²) for nauplii of *Artemia franciscana*, reported by Dattilo et al. (2005). These radiation doses were calculated using the spectroradiometer measurements.

The 24- and 48-h EC₅₀ values of TA and TM for *D. similis* and *A. salina* are provided in Table 3.

Growth tests

Daphnia similis

After 96 h of exposure, the mobility in the control groups exceeded 90 %, under both illumination condition, and the size of the organisms increased by 14.4 %. There was no statistically significant difference between the growth rates of the control groups with or without exposure to UV light (Fig. 2I, II).

Table 1 DLS measurements of 100 mg/L suspensions of TA and TM in the media used for *Daphnia* (D), *Artemia* (A), and *Artemia* with saccharose (AS)

	0 h	3 h	6 h	24 h
A				
TM				
Z-average (nm)	1407.3 (± 222.6)	1790.33 (± 163.5)	1692.6 (± 96.4)	3711.23 (± 4879.8)
PdI	0.06 (± 0.05)	0.42 (± 0.01)	0.44 (± 0.1)	0.7 (± 0.2)
Peak (nm)	1466.0 (± 257.2)	1150.6 (± 111.2)	1097.9 (± 148.5)	351.6 (± 207.4)
ZP (mV)	2.4 (± 0.9)	3.0 (± 0.9)	2.2 (± 1.0)	-7.4 (± 5.8)
TA				
Z-average (nm)	1601.6 (± 58.7)	2383.0 (± 108.5)	2444.6 (± 967.8)	1715.0 (± 980.0)
PdI	0.7 (± 0.1)	1 (± 0.0)	0.9 (± 0.08)	0.9 (± 0.1)
Peak (nm)	706.8 (± 45.9)	507.3 (± 64.0)	637.6 (± 267.2)	642.5 (± 207.5)
ZP (mV)	-1.9 (± 0.7)	-4.3 (± 1.2)	-5.6 (± 3.0)	-8.2 (± 5.5)
AS				
TM				
Z-average (nm)	1640 (± 585.6)	3602.3 (± 556.1)	3267.0 (± 614.5)	4657.5 (± 1529.4)
PdI	0.08 (± 0.1)	0.5 (± 0.09)	0.6 (± 0.09)	1.0 (± 0.0)
Peak (nm)	1689.0 (± 616.3)	1399.3 (± 445.8)	1034.6 (± 191.0)	236.3 (± 225.8)
ZP (mV)	-1.5 (± 0.8)	-1.9 (± 1.5)	-1.1 (± 1.8)	-4.0 (± 1.6)
TA				
Z-average (nm)	2108.6 (± 103.6)	2786.6 (± 397.9)	1945.6 (± 235.1)	2483.5 (± 1587.4)
PdI	0.8 (± 0.1)	0.8 (± 0.2)	0.4 (± 0.0)	0.8 (± 0.2)
Peak (nm)	743.4 (± 61.2)	721.1 (± 302.6)	1237.6 (± 78.6)	733.8 (± 691.7)
ZP (mV)	-2.8 (± 0.5)	-3.2 (± 0.7)	-3.2 (± 1.8)	-8.67 (± 3.5)
D				
TM				
Z-average (nm)	779.7 (± 8.8)	1403.0 (± 91.5)	1189.0 (± 28.5)	1373.3 (± 360.2)
PdI	0.1 (± 0.03)	0.3 (± 0.06)	0.4 (± 0.03)	0.9 (± 0.06)
Peak (nm)	825.2 (± 3.5)	1164.3 (± 84.0)	780.8 (± 19.3)	360.7 (± 92.8)
ZP (mV)	-20.1 (± 0.7)	-17.2 (± 0.2)	-21.5 (± 1.0)	-13.3 (± 2.0)
TA				
Z-average (nm)	1023.3 (± 36.5)	764.43 (± 24.4)	826.1 (± 20.8)	578.2 (± 52.9)
PdI	0.4 (± 0.05)	0.3 (± 0.06)	0.3 (± 0.1)	0.4 (± 0.1)
Peak (nm)	721.5 (± 33.0)	613.0 (± 48.8)	623.6 (± 66.0)	400.1 (± 46.0)
ZP (mV)	-19.2 (± 0.3)	-20.4 (± 3.1)	-18.9 (± 0.3)	-21.5 (± 0.7)

Average size of the particles in suspension (Z-average), polydispersion index (PdI), size of the main particle population (peak), and zeta potential (ZP). Results are presented as the mean (\pm standard deviation) of three readings

However, exposure to TM revealed an influence of concentration ($p < 0.001$) on the growth rate, which was generally higher in the groups exposed to 10 mg/L of TM. The rate of growth in the group exposed to 10 mg/L under UV light was 41 % greater than that of the UV control group ($p = 0.006$), and 45 % greater than that of the group exposed to 1 mg/L under UV light ($p = 0.003$). Comparisons among the remaining

groups revealed no statistically significant differences. Exposure to TA showed no effect on the growth rate at any concentration or under any illumination condition.

Artemia salina

In all groups, the mobility after 96 h of exposure exceeded 83 %, with the control group showing a size

Table 2 Distances of the UV lamps and doses of ultraviolet radiation (UVA and UVB) required for immobilization of 50 % (EC50) of the *Daphnia similis* after 24- and 48-h exposures

	Lamp distance (cm)	UV irradiation (mW/cm ²) radiometer	UV irradiation (mW/cm ²) spectroradiometer
24-h	33.5 (28.7–37.9)	0.5 (0.5–0.6)	1.7 (1.5–1.9)
48-h	50.9 (45.2–56.0)	0.3 (0.3–0.4)	1.0 (0.8–1.2)

The 95 % confidence intervals of the ED50 are given in parentheses. Measurements by radiometer consider the sums of the values obtained with two sensors (365 ± 2 and 313 ± 2 nm). Measurements by spectroradiometer consider the 280–400 nm range of wavelengths

increase of 119 ± 12 %. The growth rate in the control group was 20 % higher under UV light than in the absence of UV light (*p* = 0.004) (Fig. 2III). When the organisms were exposed to TM, the only statistically significant effect (*p* < 0.001) concerned the illumination condition, with increased growth rates of the organisms exposed to UV light, for both the control group (*p* < 0.001) and the group exposed to 0.06 mg/L of TM (*p* = 0.019). In the case of exposure to TA (Fig. 2IV), the statistical analysis revealed that the effect of concentration depended on the illumination condition, demonstrating that there was an interaction between the two factors (*p* = 0.045). The post-test did not identify any significant differences among the remaining groups.

Biochemical analysis in *D. similis*

When the organisms were exposed to TA, the specific activity of CAT (Fig. 3I) showed an effect of both illumination condition (*p* = 0.006) and concentration (*p* < 0.001), but there was no interaction between the two factors (*p* = 0.13). There was no difference between the controls, but the CAT activity for the group exposed to 75 mg/L under UV light was 42 % greater than for the same group under visible light (*p* = 0.002). Considering the groups that were not exposed to UV light, the CAT activity diminished by 31 % for the group exposed to 750 mg/L, compared to the control group (*p* < 0.001). The groups exposed to UV light showed a reduction of 22 and 34 % in CAT activity when exposed to 7 (*p* = 0.009) and 750 mg/L (*p* < 0.001), respect to control group. The specific activity of GST (Fig. 3II) showed no effect of nano-

Table 3 Nano-TiO₂ toxicity to *Daphnia similis* and *Artemia salina* (24- and 48-h exposures), under visible light or visible and UV light

		EC50 24-h (mg/L)		EC50 48-h (mg/L)	
		Visible light		Visible light	
		Visible light	UV light	Visible light	UV light
<i>Daphnia similis</i>	TA (anatase)	>1000.00	>1000.00	>1000.00	750.55 (599.56–1008.92)a
	TM (anatase/rutile)	>1000.00	>1000.00	>1000.00	60.16 (48.30–77.94)b
<i>Artemia salina</i>	TA (anatase)	949.07 (783.16–1269.15)a	14.40 (10.64–19.13)b	480.67 (382.18–604.24)c	4.05 (2.35–5.62)d
	TM (anatase/rutile)	945.75 (758.36–1317.90)a	16.68 (13.87–21.14)b	284.81 (213.01–374.83)e	4.03 (2.98–5.40)d

The EC50 values are given, together with the corresponding 95 % confidence intervals. Different lower case letters indicate statistically significant differences between the EC50 values for the same organism

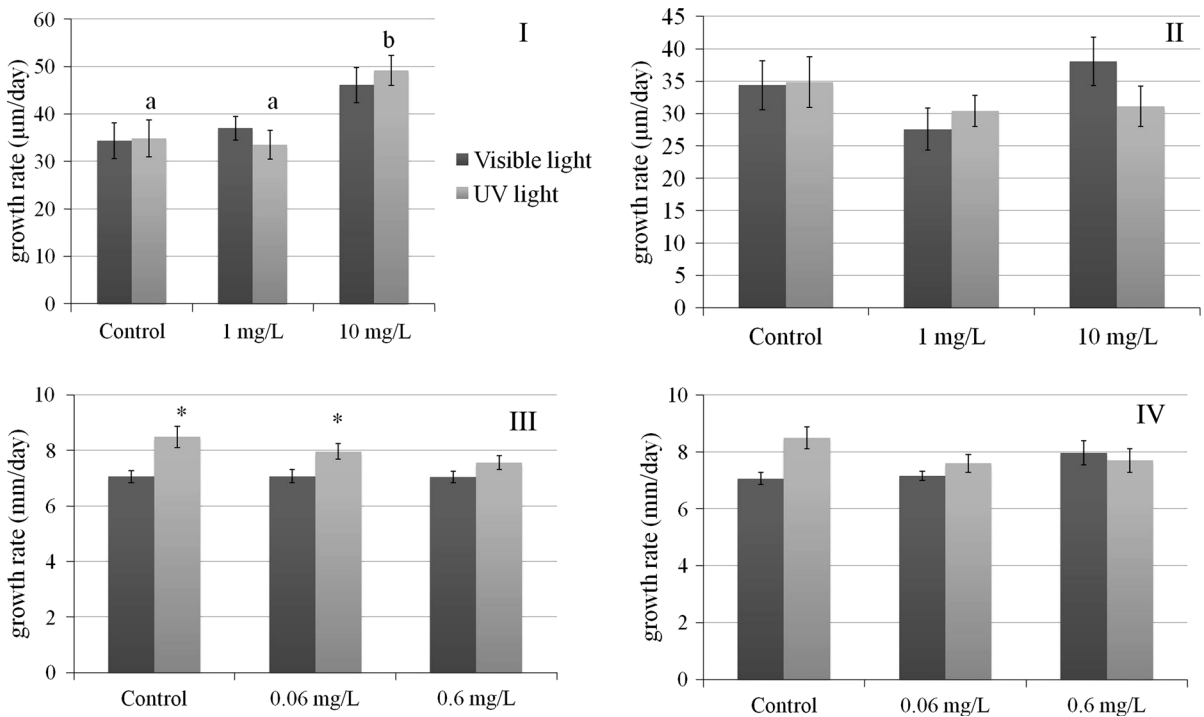


Fig. 2 Growth rates of microcrustaceans exposed for 96 h to nano-TiO₂ under visible light or visible and UV light. **I** Exposure of *Daphnia similis* to TM; **II** exposure of *Daphnia similis* to TA; **III** exposure of *Artemia salina* to TM; **IV** exposure of *Artemia*

salina to TA. Mean ± standard error. An asterisk (*) indicates $p < 0.05$ between groups exposed with and without UV, for the same concentration. Different lower case letters indicate $p < 0.05$ between groups exposed under UV light

TiO₂, but there was an effect of illumination condition ($p = 0.018$), with the activity for the control group under UV light being 22 % higher than for the control group under visible light ($p = 0.005$). The effect of the TA concentration on the specific activity of AP (Fig. 3III) depended on illumination condition, demonstrating that there was interaction between the two factors ($p = 0.017$). The AP activity differed for the controls with and without UV light ($p = 0.001$). For the groups exposed under UV, the AP activity was 20–24 % lower for those treated with 7, 75, and 750 mg/L of TA, compared to the control group ($p \leq 0.001$). No differences among the different treatment groups were observed for the specific activities of SOD and GPx (Supplementary information 4).

When the organisms were exposed to TM, the specific activity of SOD (Fig. 4) was the only biomarker that showed a response, with evidence of interaction between the TM concentration and illumination condition ($p = 0.03$). The SOD activity for the

group exposed to 0.6 mg/L under UV light was around 42 % smaller, compared to the group exposed to the same concentration without UV ($p = 0.013$). Among the groups exposed to TM under visible light, those exposed to 6 mg/L ($p = 0.001$) and 60 mg/L ($p = 0.003$) presented SOD activities that were 57 and 51 % lower, respectively, compared to the 0.6 mg/L group; however, there was no difference relative to the control. Exposure to TM had no statistically significant effect on the remaining biochemical biomarkers (Supplementary information 5).

Discussion

The UV light levels employed in the bioassays with *Daphnia* and *Artemia* corresponded to doses that were around 250 and 19 times lower, respectively, than the solar irradiation measured in spring and autumn in a subtropical region (Clemente et al. 2013). More than 10 % of the UVB radiation incident on the surface of

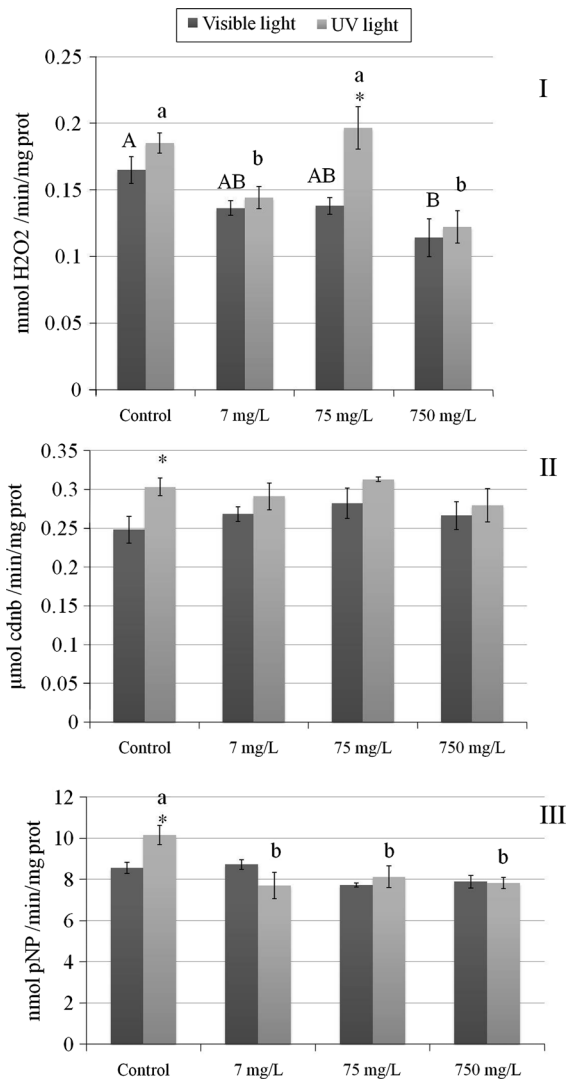


Fig. 3 Biochemical analysis in *Daphnia similis* exposed for 24 h to nano-TiO₂ (control, 7, 75, and 750 mg/L of TA), under visible light or visible and UV light. Specific activities of **I** catalase (CAT), **II** glutathione *S*-transferase (GST), and **III** acid phosphatase (AP). In all analyses, at least three samples were analyzed for each group. Mean ± standard error. An asterisk (*) indicates $p < 0.05$ between groups with and without UV, for the same concentration. Different upper case letters indicate $p < 0.05$ between different concentrations under visible light. Different lower case letters indicate $p < 0.05$ between different concentrations under UV light

clean seawater can penetrate to a depth of 15 m (Calkins 1974; cited by Acra et al. 1990). Considering that during the bioassays, the water column measured only 1 cm, and that in the environment *Daphnia* and *Artemia* can move freely in the water column, it is reasonable to suppose that despite attenuation of the

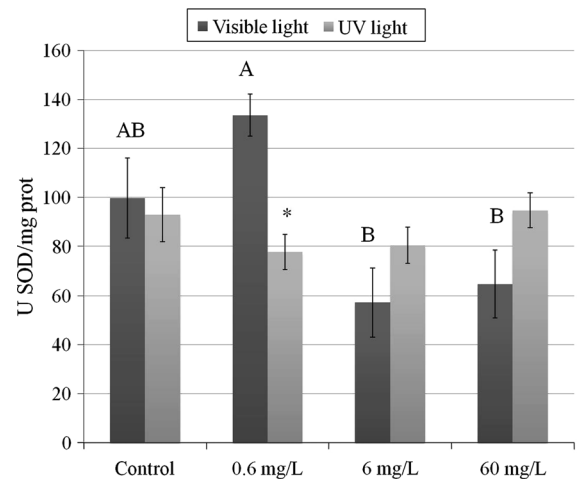


Fig. 4 Specific activity of superoxide dismutase (SOD) in *Daphnia similis* exposed for 24 h to TM under visible light or visible and UV light. Mean ± standard error. An asterisk (*) indicates $p < 0.05$ between groups exposed with and without UV, for the same concentration. Different upper case letters indicate $p < 0.05$ between groups exposed to different concentrations under visible light

UV radiation by the water, the organisms were exposed to a substantial dose of UV light, compatible to that obtained at greater depth in aquatic ecosystems (Stewart and Hopfield 1965; cited by Acra et al. 1990). During the bioassays with nano-TiO₂, UVA was the major component of UV light and UVB levels were virtually zero, but according to Ma et al. (2012a), an absence of UVB has no significant impact on the production of ROS during photocatalysis in the presence of nano-TiO₂. UVA radiation, on the other hand, appears to be fundamental for the process.

The results are in agreement with previous reports indicating that typical light conditions do not induce any acute toxicity in *Daphnia* (Hund-Rinke and Simon 2006; Lovern and Klaper 2006; Ma et al. 2012b), and that the inclusion of UV radiation in bioassays alters the nano-TiO₂ toxicity (Reeves et al. 2008; Vevers and Jha 2008; Marcone et al. 2012; Tong et al. 2013). Minimal levels of UV radiation acted to increase the nano-TiO₂ toxicity, enabling the establishment of EC₅₀ values that differed according to the formulation and the bioassay conditions. The results indicated that the EC₅₀_{24h} and EC₅₀_{48h} values exceeded 100 mg/L for *D. similis* and *A. salina*, under common conditions of illumination, hence classifying the nano-TiO₂ as being practically non-toxic to these organisms (USEPA 1985). Under the UV light exposure conditions

employed, for *D. similis*, the EC50_{48h} of the anatase/rutile mixture was around 12 times lower than that of the formulation containing pure anatase. The anatase/rutile mixture could, therefore, be classified as slightly toxic to *Daphnia*, while anatase was practically non-toxic (USEPA 1985). In the bioassays with *Artemia*, for both formulations tested, the EC50_{24h} values obtained under standard illumination conditions diminished around 60-fold when exposure to UV radiation was included. In the case of the 48-h exposures, the EC50_{48h} of the formulation containing the anatase/rutile mixture diminished around 70-fold when UV radiation was included, while that of pure anatase diminished 120-fold. Under UV radiation, the EC50_{48h} values of both formulations for *Artemia* were similar, and classified the nano-TiO₂ as being moderately toxic to *A. salina*.

As discussed previously, the anatase/rutile mixture is more photoactive than the pure crystal phases. The reasons for the different photocatalytic properties of the different TiO₂ crystal phases remain unclear. Hypotheses that have been raised include differences between the crystal phases in terms of the band gap, Fermi level, adsorption of O₂, and absorption of UV radiation (Banerjee et al. 2006; Coatingsys 2009; Sun and Xu 2010; Cong and Xu 2012). The association with the cellular membranes and cytotoxicity of nano-TiO₂ seems to be dependent on both size and crystal phase (Allouni et al. 2012; Xiong et al. 2013). Allouni et al. (2012) reported that the percentage of cells associated with nano-TiO₂ was significantly higher for particles containing the anatase/rutile mixture than for formulations containing only one of the crystal phases. Based on the available evidence, we suggest that the different responses induced in the organisms tested could have been related to the different photocatalytic properties (and, therefore, generation of ROS), and/or different rates of adsorption or absorption of the nano-TiO₂ formulations by the organisms.

Clément et al. (2013) reported that *D. magna* presented greater acute sensitivity to TiO₂, compared to other organisms tested. In the present work, *A. salina* showed to be more sensitive than *D. similis*. In general, *Artemia* is more resistant to various contaminants, compared to *Daphnia*, although exceptions have been described for substances such as arsenic and iron sulfate, whose EC50 values are lower for *Artemia* (Calleja et al. 1994). The lower nano-TiO₂ EC50 values under UV light obtained for *A. salina*, compared

to *D. similis*, could have been related to the greater UV dose employed for the first species, but the different behaviors of the NPs in the media utilized for each species could also have played a role in influencing the responses.

Azevedo et al. (2004) found that low concentrations of NaCl (2 g/L) did not affect the degradation of phenol using photocatalysis in the presence of P25[®], but that high concentrations (50 g/L) reduced the efficiency of the process by 81 %. In tests employing *Escherichia coli* (Pagnout et al. 2012), the addition of electrolytes (NaCl, CaCl₂, and Na₂SO₄) progressively reduced the toxicity of P25 at pH values below the pHzpc, while the toxicity increased for pH values above the pHzpc. Values of 6.3 have been described for the pHzpc of anatase and P25[®] (Finnegan et al. 2007; Kosmulski 2009). Such findings could explain the greater sensitivity shown by *A. salina*, given that the bioassay was performed at pH 8.7 under conditions of high salinity (30 g/L). The pH, electrolyte concentration, and valency of the ions determine the strength of electrostatic forces, influencing the balance of attraction and repulsion between the nanoparticles, as well as between the nanoparticles and the cells exposed to them.

Reactive oxygen species are capable of causing damage to biomolecules, and an increase in their production could explain the greater toxicity of nano-TiO₂ under UV light. The 24-h exposure of *Daphnia* to concentrations at and below the EC50_{48h}, under UV, resulted in different responses of the biochemical biomarkers studied. Exposure to anatase caused inhibition of the CAT and AP activities. The inhibition of CAT activity was observed in the groups exposed to 750 mg/L, with and without UV, as well as in the group exposed to 7 mg/L under UV light. In the presence of UV light, AP activity was inhibited at all the anatase concentrations tested. Exposure to TM only affected the SOD activity, which was inhibited at the lowest concentration tested (0.6 mg/L) under UV light; nonetheless, there was no statistically significant difference, compared to the control group.

The literature still remains inconclusive concerning the effects of nano-TiO₂ on biochemical biomarkers. Zhu et al. (2011) exposed marine molluscs to anatase (10 nm) for 96 h, using concentrations of up to 10 mg/L, and observed sublethal effects but no mortality. At 1 mg/L (but not at 10 mg/L), there was an increase in the activity of SOD, while at 1 and

10 mg/L there were reductions in GSH levels. Working with other aquatic organisms exposed to nano-TiO₂ has also provided evidence of reductions in the activity and expression of CAT and SOD (Hao et al. 2009; Cui et al. 2010). Inhibition of the CAT and SOD activities by a variety of contaminants, and consequent oxidative stress, has been described in several studies (Sun and Oberley 1989; Kumagai et al. 1995; Bagnyukova et al. 2005). However, it was not possible to find any report concerning the direct inhibition of these enzymes by nano-TiO₂. High concentrations of ROS, such as •O₂ and H₂O₂, are able to inhibit the activities of CAT, SOD, and phosphatases (Östman and Böhmer 2001; Lardinois et al. 1996; Gottfredsen et al. 2013). Hussain et al. (2009) found that the administration of CAT reduced pro-inflammatory responses in bronchial epithelial cells exposed to anatase and an anatase/rutile mixture, indicating that such responses were related to oxidative stress, especially the generation of H₂O₂. The occurrence of oxidative stress could be related to the direct production of ROS by the nano-TiO₂, or be a result of hypoxia caused by adherence of the nano-TiO₂ to the organisms, resulting in respiratory difficulties (Boyle et al. 2013). Exposure to UV light enhances the generation of ROS by nano-TiO₂, and in the present work, this acted to increase the toxicity of the materials to *Daphnia* and *Artemia*, in terms of both immobility and sublethal effects.

The test involving the growth of *Daphnia* has shown a good correlation with tests of chronic toxicity over periods of 14–21 days (Hanazato 1998). Sublethal concentrations of anatase did not induce any changes in the growth rate of the *Daphnia*, while at the highest concentration the anatase/rutile mixture increased the growth rate, especially under UV light. The exposure of *Artemia* to sublethal concentrations of nano-TiO₂ resulted in increased growth rates when the organisms were exposed under UV light. Furthermore, the effect of anatase concentration depended on the illumination condition, with a tendency for an increased growth rate when the anatase concentration was increased, but slower growth when the organisms were exposed under UV light. Further investigations will be necessary in order to fully understand these findings, but a possible explanation could be related to bactericidal effects of the UV radiation and the nano-TiO₂, and consequently a better state of health and improved growth of the organisms.

The literature reports contradictory results concerning the impact of nano-TiO₂ on the growth of microcrustaceans. Several authors have described a negative impact of exposure to nano-TiO₂ on the reproduction and growth of *Daphnia* (Zhu et al. 2010; Fouqueray et al. 2012; Campos et al. 2013), while other work has found an absence of any effect (Lee et al. 2009). Rozenkranz (2010) found a dose-dependent increase in the molting frequency of *D. magna* exposed to P25, with organisms exposed to 1 µg/L of P25[®] being significantly larger than those of a control group. Li (2011) found that exposure of *C. dubia* to 20 mg/L of P25 caused an increase in the organisms size, relative to the control, while exposure to 50 and 100 mg/L had negative effects on growth and reproduction.

Since the 0.6 mg/L concentrations of the formulations did not cause any changes in the growth rates, mortality, or behavior of the test organisms, in either in the presence or absence of UV light, this could be considered to be the ‘no observed effects concentration’ (NOEC) for *A. salina*. Taking the growth tests into consideration, the NOEC values for *D. similis* would be 10 mg/L for anatase (with and without UV light), and 1 and 10 mg/L for the anatase/rutile mixture, with and without UV radiation, respectively. However, the biochemical biomarkers indicated effects at concentrations different to those evaluated in the growth tests. Hence, in the case of *Daphnia*, the NOEC values may be indicated as <0.6 mg/L for anatase/rutile mixture in the presence of UV light, and 75 mg/L for anatase under visible light. These values are above the predicted environmental concentrations (PEC) of nano-TiO₂ calculated for the United States and Europe (21 ng/L for surface waters, and 4 µg/L for sewage treatment effluents) (Gottschalk et al. 2009). Nonetheless, concentrations of Ti from 185 to 2,800 µg/L have been detected in municipal sewage treatment wastewater lagoons in Arizona (Kiser et al. 2009). Given the continuing growth in the production and use of materials containing nano-TiO₂, it is possible that the presence of these substances in aquatic ecosystems could increase over time. Projected production volumes indicate that the quantity of nano-TiO₂ could increase exponentially (Robichaud et al. 2009). The changes observed in the biomarkers studied here should, therefore, serve as an alert.

Intense aggregation and precipitation of the nano-TiO₂ were observed during the bioassays, in agreement

with the literature (Sharma 2009). The characteristics of the medium, such as pH, ionic strength, and the presence of organic matter, should be taken into consideration in nanoecotoxicological tests, as well as the characteristics of the nano-TiO₂ employed. Different precipitation rates were observed, depending on the medium and the concentration and type of nano-TiO₂. Hence, although the discussion presented here has been based on nominal initial concentrations, the differences observed between the groups, in terms of the parameters evaluated, should be considered with care, since they relate to a dynamic system whose behavior still remains to be fully understood (Keller et al. 2010). The instability of the suspensions, especially at high concentration, could explain the lack of dose–response in most sublethal effects. The apparent lack of dose–response in biomarkers can also be related to the possibility that the oxidative stress in organism is related to hypoxia, more than nano-TiO₂ concentration.

In general, the precipitation rate of nano-TiO₂ was higher in the highest concentrations and *Artemia* medium, with or without saccharose. Solubility of various chemical agents in sea water is often decreased (Dvorak et al. 2012). A solution could be to reduce the salinity or use some surfactant to improve the nano-TiO₂ dispersion. It should be emphasized that in low salt concentration, *Artemia* does not consume glucose; consequently, the realization of the prolonged test is compromised. On the other side, it should be pondered the environmental relevance of use of dispersants in nanotoxicological tests.

Finally, the correct evaluation of the risk of nanotechnology requires prior understanding of all the factors involved in the behavior of different NP formulations, as well as in the generation of toxicity. The present work adds knowledge concerning the toxicity of nanoparticles of TiO₂ in two organisms that play crucial roles in freshwater and saltwater ecosystems. It was clearly evident that interpretation of the effects of nano-TiO₂ in aquatic organisms depends on the type of bioassay, the nature of the formulation (especially crystal phase), and the illumination conditions employed. The results emphasize the need to adapt ecotoxicological evaluation protocols to enable them to be used in nanoecotoxicological studies, especially in the case of nano-TiO₂, for which the behavior of the particles in the exposure medium needs to be taken into consideration, together with the photocatalytic properties of the material.

Conclusions

Determination of the nano-TiO₂ toxicity using bioassays depends on the organism, culture medium, and exposure time employed. It also depends on the crystal phase and the illumination conditions. Exposure to UV radiation at minimal environmental levels increases the nano-TiO₂ toxicity. *A. salina* showed greater acute sensitivity to nano-TiO₂, compared to *D. similis*, in either in the presence or absence of UV light. Under UV light, the anatase/rutile mixture was more toxic to *D. magna*, compared to pure anatase. For *A. salina*, the two crystal phases only showed different effects when the exposure was performed in the absence of UV light, with the mixture being more toxic than pure anatase. The acute exposure of *Daphnia* to concentrations at and below the EC50_{48h} of nano-TiO₂, under UV irradiation, inhibited the specific activities of CAT, SOD, and AP, indicating the occurrence of oxidative stress. At sublethal concentrations, the nano-TiO₂ did not show any negative impacts on the growth of *Daphnia* and *Artemia*. The results indicated that the nano-TiO₂ could potentially have negative impacts in freshwater and saltwater ecosystems. The findings contribute to the discussion of nanoecotoxicological protocols and their implementation.

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