Ecotoxicology of Nano-TiO$_2$—An Evaluation of its Toxicity to Organisms of Aquatic Ecosystems

Clemente, Z.$^{1,2,*}$, Castro, V. L.$^2$, Jonsson, C. M.$^2$ and Fraceto, L. F.$^{1,3}$

$^1$ Department of Biochemistry, Institute of Biology, State University of Campinas – UNICAMP, Rua Monteiro Lobato, 255, CEP 13083-862 Campinas, SP, Brazil
$^2$ Laboratory of Ecotoxicology and Biosafety, Embrapa, Rodovia SP340 km 127.5, C.P. 69, CEP 13820-000 Jaguariúna, SP, Brazil
$^3$ Department of Environmental Engineering, São Paulo State University – UNESP, Avenida Três de Março, 511, CEP 18087-180 Sorocaba, SP, Brazil

ABSTRACT: The production and use of synthetic nanoparticles is growing rapidly, and therefore the presence of these materials in the environment seems inevitable. Titanium dioxide (TiO$_2$) presents various possible uses in industry, cosmetics, and even in the treatment of contaminated environments. Studies about the potential ecotoxicological risks of TiO$_2$ nanoparticles (nano-TiO$_2$) have been published but their results are still inconclusive. It should be noted that the properties of the diverse nano-TiO$_2$ must be considered in order to establish experimental models to study their toxicity to environmentally relevant species. Moreover, the lack of descriptions and characterization of nanoparticles, as well as differences in the experimental conditions employed, have been a compromising factor in the comparison of results obtained in various studies. Therefore, the purpose of this paper is to make a simple review of the principal properties of TiO$_2$, especially in nanoparticulate form, which should be considered in aquatic toxicology studies, and a compilation of the works that have been published on the subject.

Key words: Nano-TiO$_2$, Nanotechnology, Ecotoxicology, Water, Aquatic organisms

INTRODUCTION

Nanotechnology is a rapidly expanding area of research which already has a wide variety of commercially available products. The material most commonly utilized in nanoproducts is silver, followed by carbon, titanium, silicon, zinc and gold (Meyer et al., 2009, Project on Emerging Nanotechnologies, 2009). An initial estimate indicates that nanotechnology may lead to a revolution in the development and fabrication of products that could contribute with up to one trillion dollars to the global economy by 2015 (Roco, 2001). Nanomaterials have dimensions of less than 100 nanometers (nm), while nano-objects have dimensions smaller than 100nm and nanoparticles (NPs) have three dimensions with less than 100 nm (Stone et al., 2010). However, the literature often describes NPs as particles that possess at least one dimension in the order of 1 to 100 nanometers (nm). The Royal Society of Chemistry suggests that 100 nm is the cut-off point above which particles will not enter cells through receptor-mediated processes (RSCRAE, 2005), and some experimental evidence has emerged that corroborates this dimension.

*Corresponding author E-mail: zairaclemente@hotmail.com

(Chithrani and Chan, 2007, Clift et al., 2008). Another important cut-off dimension is particles smaller than 40 nm, which can enter the nucleus, while particles smaller than 35 nm can, potentially, cross protective barriers such as the hematoencephalic barrier (Oberdorster et al., 2004). However, these values should serve as guidelines, since the real size to be considered depends on other factors of the material and on details of its surface.

Titanium dioxide (TiO$_2$) has been used commercially since 1900, particularly in coatings and pigments. In 2002, the production capacity of this oxide was estimated at 4.6 million tons (Winkler, 2003). A review published by the United States Environmental Protection Agency (USEPA) estimated the annual production of TiO$_2$ nanoparticles (nano-TiO$_2$) to be 2000 metric tons in around 2005, with 65% of this production used in products such as cosmetics and sunscreen lotions (USEPA, 2009). The growing use of NPs generates effluents or wastewaters, raising concerns about the environmental risks and impacts...
of nanotechnology. Due to the wide utilization and promising uses that have emerged from nano-TiO₂, this material has been the target of several ecotoxicology studies. Based on a compilation of publishes works that evaluate the toxicity of nano-TiO₂ to aquatic organisms, the article reviews the main properties of TiO₂, especially in nanoparticulate form, which should be considered in aquatic toxicology studies.

In nature, TiO₂ occurs only in the form of oxide or oxides mixed with other elements. Mineral deposits are usually of volcanic origin, but are also found in beach sand (Winkler, 2003). TiO₂ can be found in three crystalline forms: anatase (tetragonal), rutile (tetragonal) and brookite (orthorhombic), and its main reserves are located in Canada, the US, Scandinavia, South Africa, the Mediterranean Sea, and Australia (Titaniumart, 2010). Titanium dioxide, also known as titanium oxide (IV) or titania (molecular weight 79.88), is insoluble in water, chloric acid, nitric acid and ethanol, but is soluble in concentrated and heated sulfuric, hydrogen fluoride and alkaline media (NRC, 1999).

TiO₂ is obtained mainly from ore containing ilmenite (FeTiO₂), natural rutile (TiO₂) and leucoxene-like ilmenite. TiO₂ particles are referred to as primary, aggregates or agglomerates. Primary particles are individual crystals bound by crystal planes. Agglomerates are sintered primary particles connected by their crystal faces. Agglomerates are multiple primary particles and aggregates that are joined together by van der Waal forces (IARC, 2010). Primary particles typically have a diameter of 0.2 to 0.3 μm, although larger aggregates are also formed (further details about bulk TiO₂ are given in Diebold, 2003). Several TiO₂ NPs are produced today (Xiaobo, 2009), with variations in particle size, surface area, purity (due to doping, coating or quality control), surface characteristics, crystalline shape, chemical reactivity and other properties. One of the main differences between bulk TiO₂ and nano-TiO₂ is the larger surface area of a given mass or volume of NPs compared to an equivalent mass or volume of bulk TiO₂ particles (Shao and Schlossman, 1999). Approximately 35-40% of atoms are located on the surface of a 10 nm NP compared with less than 20% on particles larger than 30 nm. This higher surface area reinforces several properties, such as photocatalytic activity and ultraviolet absorption at given wavelengths (Shao and Schlossman, 1999). Bulk TiO₂ absorbs ultraviolet radiation (<400nm). Because of its high refractive index, it is also very effective in dispersing radiation. Both dispersion and absorption are important in the attenuation of ultraviolet radiation (UV), making it an effective ingredient in sunscreen lotions (USEPA, 2009). Small primary particles are less able to disperse visible light and are more transparent, while larger size particles are more opaque. Hence, sunscreen formulations containing nano-TiO₂ have become popular due to their greater transparency on the skin compared to the white appearance of formulations containing bulk TiO₂.

The theoretical calculations of Palmer et al. (1990) and experimental data of Sakamoto et al. (1995) showed that the UVB attenuation of submicrometric TiO₂ particles is predominantly due to their absorption, while UVA attenuation is essentially due to their dispersion. The findings of Shao and Schlossman (1999) contribute to the idea that smaller particle sizes, and hence larger specific surface areas, are better for UVB attenuation. In contrast, the intensity of UVA dispersion is greater the larger the particle size (Shao and Schlossman, 1999). TiO₂ is a semiconductor, i.e., a crystalline solid whose electrical conductivity is intermediate between that of conductors and insulators. Thus, an important application of this material is in the electronics industry and in processes of heterogeneous photocatalysis.

The principle of heterogeneous photocatalysis involves the activation of a semiconductor by solar or artificial radiation. A semiconductor is characterized by two energy regions: the region of lower energy is the valence band (Eᵥ), where the electrons cannot move freely, and the higher region is the conduction band (Eᶜ), where the electrons move freely through the crystal, producing electrical conductivity similar to that of metals. These two regions are divided by a “band-gap” zone. Fig. 1 shows a schematic representation of a semiconductor particle. The absorption of photons with energy higher than the band-gap energy (Eᵥ) causes the promotion of an electron from the Eᵥ to the Eᶜ, with the concomitant generation of a gap (h⁺) in the Eᵥ. In the absence of suitable scavengers species, the stored energy is dissipated within milliseconds by recombination, with the formation of an unpaired electron. If a suitable scavenger or a surface defect is available to contain the electron or gap, recombination is prevented and redox reactions occur subsequently. Eᵥ gaps are potent oxidants (potential of +1.0 to +3.5 V, depending on the semiconductor and pH) that are able to generate radical species (HO•, O₂•, H₂O₂, •etc.) from water molecules adsorbed on the semiconductor surface, which can subsequently oxidize other molecules (Nogueira and Jardim, 1998, Gaya and Abdullah, 2008, Malato et al., 2009). There are indications that the reaction occurs only in the adsorbed phase of the semiconducting particle, hence, organic molecules that can effectively adhere to the surface of the photocatalyst are more susceptible to direct oxidation (Gaya and Abdullah, 2008).

The minimum Eᵥ required for a photon to cause the photogeneration of charged species in TiO₂
(anatase form) is 3.2 eV, which corresponds to a wavelength of 388 nm. In fact, the photoactivation of TiO$_2$ occurs in the range of 300-388nm (Nogueira and Jardim, 1998, Gaya and Abdullah, 2008). Thus, the strong resistance of TiO$_2$ to decomposition and photocorrosion, its low cost, and the possibility of using solar UV radiation, makes it particularly interesting for processes of heterogeneous photocatalysis (Malato et al., 2009).

Many studies have demonstrated the potential use of heterogeneous photocatalysis with TiO$_2$ for the degradation of organic and inorganic compounds (Chatterjee and Dasgupta, 2005, Fujishima and Zhang, 2006). For the most part, photodegradation leads to the total mineralization of pollutants, generating CO$_2$, H$_2$O and inorganic acids (Malato et al., 2009). This property is applicable in the production of self-cleaning surfaces, cleaning products, in the remediation of contaminated soil and water, or even the deodorization of environments and the destruction of gas-phase volatile compounds. The hydroxyl radicals generated during TiO$_2$ irradiation are also able to react with most biological molecules, resulting in bactericidal and virucidal activity (Nogueira and Jardim, 1998, Li et al., 2008).

Studies suggest that anatase and rutile have different photocatalytic properties, with anatase possessing the better combination of photoactivity and photostability (Gaya and Abdullah, 2008, USEPA, 2009). The rutile form is inactive for the photodegradation of organic compounds, although the reason for this is not completely clear (Nogueira and Jardim, 1998, Malato et al., 2009). However, the low adsorption capacity of O$_2$ on its surface is pointed out as one of the possible factors.

Among the different titanium oxide products, TiO$_2$ P25 fabricated by Evonik Degussa Corp. (Germany) is the one most commonly used because of its reasonably well defined nature (typically a mixture of 70:30 anatase:rutile, nonporous, surface area of about 50 m$^2$/g, and average particle size of 30 nm) and its high photoactivity when compared to that of other sources (Nogueira and Jardim, 1998, Malato et al., 2009).

Surface treatment of nano-TiO$_2$ can alter its light absorption and photocatalytic activity. In applications such as paints, coatings and cosmetics, which require chemical stability, the photocatalytic properties of TiO$_2$ are generally suppressed by coatings it with silica and aluminum layers (Diebold, 2003, Li et al., 2008). Doping of nanostructured TiO$_2$ materials has also often been employed to modify its band-gap energy and increase its photocatalytic activity. TiO$_2$ is generally used in suspension (also called slurry), but can also be used immobilized in an inert matrix coating surfaces (Gelover et al., 2006, Gaya and Abdullah, 2008, Malato et al., 2009).

Immobilized TiO$_2$ has been reported to have low catalytic activity when compared to systems in suspension (Gaya and Abdullah, 2008, Malato et al., 2009). The mineralization rate generally increases with the concentration of the catalyst up to a limit of high concentration. Wei et al. (1994) used P25 for the disinfection of E. coli in water and reported that the disinfection rate depended mainly on two variables: the intensity of incident light and the TiO$_2$ dose.
In general, for any photocatalytic application, the optimal concentration should be determined in order to avoid an excess of catalyst and to ensure the total absorption of photons, i.e., to ensure the entire exposed surface of the particles is illuminated. When the concentration of TiO$_2$ is too high, the turbidity prevents radiation from penetrating and reaching all the particles (Herrmann, 1999). In photocatalysis studies, the optimal of TiO$_2$ have been a temperature of 20 to 80°C, a concentration of 200-500 mg/L, oxygen concentration of $pO_2 \geq 0.21$ atm and pH preventing pHzpc (Malato et al., 2009).

NPs tend to aggregate in the environment and can therefore be eliminated or captured by sedimentation. NP aggregates are generally less mobile and can interact with filtering organisms and with organisms that feed on sediment, or even with suspended organic matter. It is therefore important to understand the behavior of TiO$_2$ NPs in aquatic environments in order to understand their toxicology. The pH, ionic concentration and nature of the electrolytes in aqueous suspensions have been reported as important parameters in the aggregation of nano-TiO$_2$ (Sharma, 2009).

The pH of aqueous solutions significantly affects TiO$_2$, including the particle charge, the size of aggregates and the position of the $E_c$ and $E_v$. The pH at which the surface of an oxide has no electrical charge is defined as the zero point charge (pHzpc). The pHzpc of nano-TiO$_2$ varies from 4.5 to 7, depending on the particle’s size and crystal shape, with smaller particles presenting lower pHzpc (Kosmulski, 2002 cited by Sharma, 2009). Finnegan et al. (2007) reports pHzpc values of ~5.9 for rutile and of ~6.3 for anatase. A pHzpc of 6.3 has been reported for Degussa P25 (Kosmulski, 2009).

The surface of titanium will remain positively charged in an acid medium and negatively charged in an alkaline medium (Gaya and Abdullah, 2008). The lack of surface charge renders an electrostatic potential null, because it does not produce the repulsive interaction needed to separate the particles in the liquid. Therefore, TiO$_2$ particles tend to aggregate close to the pHzpc.

Particle aggregation interferes in the ability of the suspension to transmit or absorb radiation. However, this variation in particle size may be an advantage when the objective is to separate TiO$_2$ from water (by sedimentation and/or filtration) at the end of a photocatalytic treatment (Malato et al., 2009).

Like other NPs, nano-TiO$_2$ can bind to organic matter, thus modifying its properties and behavior. The adsorption of acid fulvic and humic acid on nano-TiO$_2$ has proved to be pH-dependent and favors the dispersion and suspension of these particles in aquatic environments (Domingos et al., 2008, Yang et al., 2009). On the other hand, the adsorption of oxalic acid appears to destabilize nano-TiO$_2$ suspensions, increasing the sedimentation rate at pH 2, although no change in the sedimentation rate has been observed at pH 6.5 (Pettibone, 2008).

The adsorption of organic matter on nano-TiO$_2$ may also alter the adsorption of toxic compounds (Sharma, 2009). Nano-TiO$_2$ has been reported to show adsorption behavior toward metals such as Cu(II), Cr(III), Mn(II), Ni(II), Zn(II), Cd(II), Mo(VI) (Kaur and Gupta, 2009). When an aqueous suspension of bacteria and other microorganisms is in the presence of TiO$_2$, in the dark, a slight reduction in the concentration of colonies can be observed due to the possible agglomeration of TiO$_2$ with the bacterial cells and subsequent sedimentation (Malato et al., 2009).

**STUDIES OF THE AQUATIC ECOTOXICOLOGY OF TiO$_2$**

NPs differ from bulk particles in terms of their heterogeneous size distribution, surface charge, composition, degree of dispersion, etc. Therefore, in a toxicology study, it is important to determine not only their exposure concentration but also other measures (Hasselov et al., 2008). At the NanoImpactNet Workshop held in 2008, a list was proposed of the six principal characteristics of nanomaterials to be discriminated in environmental studies: size, dissolution/solubility, surface area, surface charge and surface chemical composition. Information such as size distribution, crystal structure, morphology, agglomeration/dispersion, etc. may also be important (Stone et al., 2010). Nonetheless, the authors recognize that the characterization of nanomaterials may be time-consuming and costly, as well as complex, and therefore its application should depend on the objectives of the study (Stone et al., 2010). It was also agreed that the properties should be characterized in test systems and not in the “bottles” that are supplied, and that certain properties such as agglomeration and dissolution should be listed as “rates” rather than “states” in view of the dynamic nature of nanoparticulate systems.

Unfortunately, methods to measure all the properties are not available. For example, there is still no method available to measure the surface area in an aqueous dispersion of NPs. Moreover, there is still a paucity of information about the extent to which the limitations of the different methods may influence the correct interpretation of results. The bias of a technique can be reduced by using multiple techniques, although this is difficult due to time and cost constraints (Stone et al., 2008).
et al., 2010). Hasselöv et al.’s paper (2008) presents information about the main methods available for the characterization of NPs.

The fact that TiO$_2$ is highly insoluble, non-reactive with other materials, thermally stable, and non-flammable enabled it to be declared innocuous to the organism (WHO, 1969). However, studies have demonstrated an apparently species specificity in the generation of lung tumors in rats that inhaled TiO$_2$ for long periods (Hext et al., 2005). In addition, other significant data in the literature confirm the occurrence of lung inflammation, oxidative stress and involvement of other organs after respiratory and oral exposure to nano-TiO$_2$ (Ferin et al., 1992, Wang et al., 2007, Warheit et al., 2007a). Recently, the International Agency for Research on Cancer (IARC) classified TiO$_2$ as “possibly carcinogenic for humans” (IARC, 2010).

The various possible sources of contamination of water bodies by nano-TiO$_2$ make it essential to assess its effects on ecosystems, i.e., its ecological, public health and economic consequences. There is still a paucity of studies about the presence of nano-TiO$_2$ in the environment. Natural TiO$_2$ NPs have been found in river water (Wigginton et al., 2007). In Switzerland, due to the climatic conditions, researchers reported nano-TiO$_2$ particles peeling off painted façades and being carried into surface waters, Ti concentrations of about 16 µg/L were found in urban runoff (Kaegi et al., 2008).

Nanoeutotoxicology studies are relatively recent, the first publication involving an assay with fishes dated 2004 (Orberdorster, 2004). Tables 1 to 3 summarize published works about the effects of TiO$_2$ NPs on aquatic organisms.

With regard to the bioavailability of nano-TiO$_2$ to aquatic organisms, the literature is still inconclusive. In a recent paper, Johnston et al. (2010) did not observe significant absorption of nano-TiO$_2$ in Oncorhynchus mykiss exposed for 10 days to concentrations of up to 5 mg/L. Federici et al. (2007) also did not find accumulation of nano-TiO$_2$ in O. mykiss exposed for 14 days to concentrations of up to 1 mg/L. On the other hand, some studies report that the nano-TiO$_2$ present in water may accumulate in Cyprinus carpio, Danio rerio and Daphnia magna, even at concentrations of 0.1 and 1 mg/L, although low factors of bioconcentration were determined (Zhang et al., 2006, Zhu et al., 2010a, b). Zhu et al. (2010a) report the occurrence of trophic transfer of nano-TiO$_2$ in D. rerio fed with contaminated daphnids, but discard the possibility of biomagnification. Other studies have shown that the presence of nano-TiO$_2$ may elevate the absorption of other contaminants in fishes, such as As and Cd (Sun et al., 2007, 2009, Zhang et al., 2007).

The results of toxicity tests have usually been expressed as lethal (LC$_{50}$), effective or inhibitory (EC$_{50}$) concentrations that cause, respectively, mortality, abnormality of inhibition to 50% of the exposed organisms. A wide variability has been found in the results reported in the literature with regard to toxicity tests. This variability may be due to the different characteristics of nano-TiO$_2$ and treatments applied, as well as to experimental designs. Thus, exhaustive discussion has focused on the need for the proper characterization of NPs under study, and for the standardization of nanoeutotoxicological evaluation methods. The lack of information in some works makes it difficult to compare results (Warheit et al., 2008). Discussions have also focused on the lack of analytical techniques for the characterization of NPs in the media utilized for ecotoxicological assays.

Lovern and Kapler (2006) reported an LC$_{50}$ of 5.5 ppm in D. magna exposed for 48 h to filtered nano-TiO$_2$, but did not observe mortality or behavioral abnormalities after exposure for the same period to concentrations of up to 500 ppm of the same nano-TiO$_2$, although the suspension was sonicated. Although several authors considered acute exposure to nano-TiO$_2$ of low toxicity to Daphnia (Warheit et al., 2007b, Griffith et al., 2008, Heinlaan et al., 2008, Lee et al., 2009, Strigul et al., 2009, Wiencz et al., 2009, Kim et al., 2010, Rosenkranz, 2010), prolonged exposure has presented varied results. The exposure of D. magna to Degussa P25 (sonicated) for 21 days showed a LC$_{50}$ of 2.62mg/L and alteration of the reproduction and growth rates (EC$_{50}$ 0.46 mg/L) (Zhu et al., 2010b), while exposure for the same period to different types of BASF nano-TiO$_2$ (sonicated) did not cause mortality but reduced the reproductive capacity (EC$_{50}$ 26.6 mg/L) (Wiencz et al., 2009). Kim et al. (2010) did not find reproductive impairment but reported a 70% mortality rate in D. magna exposed for 21 days to 5 mg/L of Sigma Aldrich nano-TiO$_2$.

Some studies appear to suggest that nano-TiO$_2$ has low acute toxicity for fishes, and LC$_{50}$ is indicated as 124,5 mg/L for D. rerio (Xiong et al., 2011) and >100 mg/L for O. mykiss (Warheit et al., 2007b). Similarly, the exposure of D. rerio eggs to nano-TiO$_2$ for 96 hours at concentrations of up to 500 mg/L did not cause alterations in the survival and hatching rates, or malformations (Zhu et al., 2008). The exposure of embryos of Pimephales promelas to concentrations of up to 1mg/L for 7 days also caused no significant mortality or observable malformations (Jovanovic et al., 2011). On the other hand, some studies have shown that the prolonged exposure of fish to concentrations of 1 to 200 mg/L did not cause mortality, but observed dose-dependent elevation of the respiratory rate and
swimming behavior, as well as increased production of mucus (Federici et al., 2007, Hao et al., 2009).

Evidence of adverse effects of a given contaminant at sublethal concentrations is extremely important in environmental risk assessment, since it may generate a cascade effect with consequences at the level of individuals, communities and the ecosystem. Thus, the use of biomarkers in risk assessments offers the advantage of allowing for the detection of potentially toxic exposure well before real adverse effects occur (Nascimento et al., 2008, Prospéri and Nascimento, 2008).

Studies have shown that the toxicity of some nanomaterials such as TiO₂ may be implicated in the generation of reactive oxygen species (ROS) (Kahru and Dubourguier, 2009, Pelka et al., 2009, Sharma et al., 2009). ROS can react with the majority of biomolecules and damage lipids, proteins and nucleic acids (Valavanidis et al., 2006).

Exposure in aqueous media appears to be more severe than via the diet for O. mykiss (Handy et al., 2008). The prolonged exposure of fish to nano-TiO₂ induced biochemical and histopathological alterations in their gills, liver and intestines (Federici et al., 2007, Hao et al., 2009, Johnston et al., 2010, Palaniappan and Pramod, 2010). Exposure to nano-TiO₂ can trigger oxidative stress in D. magna, fishes and mollusks (Federici et al., 2007, Hao et al., 2009, Canesi et al., 2010a, Kim et al., 2010, Xiong et al., 2011). Lysosomal instability has also been reported in polychaetes and mollusks exposed to nano-TiO₂ (Canesi et al., 2010a, Galloway et al., 2010). The intravenous administration of high doses of nano-TiO₂ in fish has shown that it accumulated in the kidneys, with slow depuration, but no significant alterations were observed in the function of this organ (Scown et al., 2009). An experiment with D. magna showed that even after a period of 72 hours in clean water, the depuration of adsorbed TiO₂ was not complete (Zhu et al., 2010b).

With regard to genotoxicity in aquatic organisms, nano-TiO₂ presents controversial results. Nano-TiO₂ has presented genotoxicity in some studies (Griffith et al., 2009, Galloway et al., 2010, Jovanovic et al., 2011) but not in others (Lee et al., 2009). Griffith et al. (2009) reported that exposure to nano-TiO₂ altered the expression of 171 genes in D. rerio involved mainly in ribosome structure and activities, but not in the regulation of oxidative stress. Jovanovic et al. (2011) also observed upregulation of genes involved in inflammatory response (especially in phagocytic processes), and suppression of neutrophil function in fish that received an intraperitoneal dose of nano-TiO₂. The immune system also appears to be an important target of TiO₂ NPs in bivalves (Canesi et al., 2010b).

In bioassays with aquatic organisms, the circadian cycle is usually established using fluorescent lamps. These lamps emit basically visible light, while in natural conditions these organisms are exposed to solar radiation (infrared, visible and ultraviolet light). There is ample evidence of the formation of reactive oxygen species when TiO₂ is exposed to UV radiation (Brezova et al., 2005). Several studies have reported the phototoxic effects of TiO₂ normal or NPs), and its consequent use in the disinfection of water (Wei et al., 1994, Carp et al., 2004, Adams et al., 2006). The photocatalytic properties of nano-TiO₂ can augment its toxic effects in aquatic organisms under environmental conditions, but few studies so far have taken this into consideration. In vitro studies have shown that co-exposure to nano-TiO₂ and ultraviolet radiation increases cyto- and genotoxicity in fish cells (Reeves et al., 2008, Vegers and Jha, 2008). The pre- and co-illumination of nano-TiO₂ has also been shown to elevate its toxicity in daphnids (Hund and Rinke, 2006, Marcone et al., 2010).

There are still uncertainties about the characterization of exposure to nanoparticles in the testing systems of all ecotoxicity assays except those that involve the oral administration of nanoparticles. These uncertainties include how the substance is dosed and maintained in the test medium, the measurement and characterization of NPs in the test system, the understanding of the abiotic factors that influence the behavior of NPs in the test system, and a consensus about the dosimetry (Crane et al., 2008).

Today there are several guidelines for conducting ecotoxicological assays (OECD, USEPA, DIN Standards, etc.). However, their use for nanoeotoxicological assays is still under question (Stone et al., 2010). The use of these methodologies must be evaluated for each type of nanoparticle. Testing with nano-TiO₂ presents various particularities, such as its photocatalytic properties and absorption of UV radiation, its aggregation and sedimentation behavior in water and its interaction with organic matter. Performing assays to determine lethal and effective concentrations in the proposed ranges of concentration is particularly difficult. The OECD, for example, suggests finding the CL₅₀ up to the concentration of 100mg/L, however, nano-TiO₂ forms a whitish suspension when dissolved in water, and in concentrations equal to or higher than 10mg/L, it precipitates rapidly if no dispersion method is used. Wiench et al. (2009) found that TiO₂ does not disperse well at 10-100 mg/L and that sedimentation occurs within 24-48 hours. For uncoated TiO₂ (BASF, >99%, 70% anatase, 30% rutile, 20-30nm, 48.6 m²/g), the
Table 1. Summary of papers published about the effects of nano-TiO₂ used in toxicology studies on microcrustaceans (Continues)

<table>
<thead>
<tr>
<th>Test species</th>
<th>Product tested</th>
<th>Treatment of the product</th>
<th>Physicochemical characterization</th>
<th>Bioassay</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. magna</em> (Kim et al., 2010)</td>
<td>Sigma Aldrich nano-TiO₂ (40 nm, 30% rutile, 70% anatase)</td>
<td>10% solution in water with pH 2 (without sonication)</td>
<td>N4 and DLS sediment profile analyzer.</td>
<td>Acute assay 48h. Without feeding during the test. USEPA 1993. Chronic assay, semi-static, 21 days. Renewal of medium and daily feeding. Concentrations tested: 0, 1, 2, 5, 10 mg/L. Evaluations made of SOD, GPX, CAT and GST activity in groups exposed for 5 days to 0, 0.5, 1, 2.5, 5, and 10 mg/L of TiO₂. GPX and GST were also tested after fractionation of the nanoparticles (&lt;200, &lt;400, and &lt;800 nm).</td>
<td>Acute assay: mortality did not reach 50% even at 10 mg/L so the LC₅₀ could not be determined. Chronic assay: highest mortality at 5 and 10 mg/L (70 and 80%, respectively). No reproductive impacts observed. Increase in CAT at 10 mg/L, no difference in SOD, GPX, highest GST increases at 5 and 10 mg/L. TiO₂ was found in the intestines of daphnids and glued to their antennae and external surface.</td>
</tr>
<tr>
<td><em>D. magna</em> (Rosenngranz, 2010)</td>
<td>Degussa P25 nano-TiO₂ 100 mg/L solution was prepared in culture medium for daphnids</td>
<td>- sonication (30 min). The remaining solutions were made from serial dilutions of 1:10.</td>
<td>INA</td>
<td>Acute assay 48h. No food during the test. 100, 10, 1 and 0.1 mg/L. Chronic assay 21 days. Medium changed daily. Daily feeding. Concentrations: 0.001, 0.1 and 1 mg/L.</td>
<td>Acute assay: 10% mortality at 100 mg/L. High molt frequency, dose-dependent. Chronic assay: high molt frequency only on the first day of exposure, at 1 mg/L.</td>
</tr>
<tr>
<td><em>D. magna</em> (Zhu et al., 2010b)</td>
<td>Degussa P25 nano-TiO₂ (21 nm, 50m²/g, 20% rutile, 80% anatase) Size of aggregates in culture medium: 1h - 580.5 nm; 12h – 2349.0 nm; 24h – 3528.6 nm</td>
<td>Stock solution (1 g/L) in ultra pure water – sonication (10 min, 50 W/L, 40kHz) new sonication (10 min, 50 W/L, 40kHz) prior to dilution in culture medium for daphnids.</td>
<td>SEM, DLS, ICP-OES (concentration of Ti in the solution and in daphnids).</td>
<td>Acute assay 72h. semi-static OECD 202. Medium renewed daily. No food during the test. Concentrations tested: 0.1, 0.5, 1, 5.10 and 50 mg/L. Chronic assay 21 days semi-static OECD 211. Daily renewal of medium and daily feeding. Concentrations tested: 0.1, 0.3, 1 and 5 mg/L. Bioaccumulation and depuration test 24h of accumulation (samples were collected at 0, 2, 6, 12 and 24h) and 72h of depuration (samples were collected at 6, 12, 24, 48 and 72h). Concentrations tested: 0.1 and 1 mg/L with and without daily feeding.</td>
<td>Acute assay: In 48h: NOEC &lt;0.01 mg/L; EC₅₀ &gt;100 mg/L. LC₅₀ &gt;100 mg/L. In 72h: NOEC &lt;0.01 mg/L; EC₅₀ = 1.62 mg/L; LC₅₀ = 2.62 mg/L. Chronic assay: At 0.1 mg/L reproduction declined. At 0.5 mg/L reproduction and growth were inhibited. Mortality was recorded in groups 1 and 5 mg/L after 8 days of exposure. EC₅₀ = 0.46 mg/L; LC₅₀ = 2.62 mg/L. The feeding rate decreased as the exposure concentration increased. Biocompatibility test Group 0.1 mg/L: Concentration plateau in 12 h, BCF = 5.4x10⁸ L/mg. time to accumulate 50% of saturation level = 3.87h; time to reach 50% separation = 26.36h. Group 1 mg/L: Concentration plateau in 24h, BCF = 118 L/mg. time to accumulate 50% of saturation level = 3.72h; time to reach 50% separation = 74.52h. Depuration was not complete, 30% of the saturation concentration remained in the daphnids at the end of the experiment. Feeding during exposure to TiO₂ increased the accumulation time and reduced the depuration time.</td>
</tr>
</tbody>
</table>
Table 1. Summary of papers published about the effects of nano-TiO$_2$ used in toxicology studies on microcrustaceans (Continues)

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |

| D. magna and | Sigma Aldrich nano-TiO$_2$ (7nm) | Solution (1mg/L) in culture medium \* sonication (15 min). | TEM, BET | Acute assay 96h. OECD 1994, 1998. Concentration tested: 1mg/L | No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group. |
| Chloromonera riparius (Yeata) | (0.040mg/g) and 20 nm \* (0.604mg/g) | | | |
Table 1. Summary of papers published about the effects of nano-TiO$_2$ used in toxicology studies on microcrustaceans

<table>
<thead>
<tr>
<th>D. magna</th>
<th>Nano-TiO$_2$, 30nm (in suspension)</th>
<th>THF was used to ensure dispersion. The THF was eliminated by evaporation and filtration and confirmed by spectrophotometry.</th>
<th>TEM: Characterization according to Levern and Klaper (2006).</th>
<th>Acute assay: 60 min. USEPA 23. Concentration total: 2ppm (LOEC calculated in a previous experiment).</th>
<th>TiO$_2$ did not significantly alter the host rate, jump, movement of appendages, and curvature of the abdominal claw.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. magna</td>
<td>DuPont HafCel TiO$_2$, fine TiO$_2$ (30nm in water: 5.5mg/L, 100% rutile, 99%TiO$_2$ and 1% aluminum), d(C(140-444 nm in water: 38.5mg/L), 9% rutile; 21% anatase, 90%TiO$_2$) and 5% amorphous silica (1% aluminum).</td>
<td>INA</td>
<td>DL, BET, X-ray fluorescence, X-ray diffraction.</td>
<td>Acute assay: 48h, static. OECD 212. Concentration total: 0.1, 1, 10 and 100 mg/L.</td>
<td>LC$_{50}$: &gt;100mg/L for both types of TiO$_2$. There was 10% of immobility at concentrations of 10 and 100mg/L at the end of 48h for both compounds tested.</td>
</tr>
<tr>
<td>D. magna</td>
<td>Sigma Aldrich nano-TiO$_2$, 65nm, 950nm, and 44µm. Smaller particles (65nm) appeared larger (on average 320nm) and larger ones (950nm and 44µm) appeared smaller (30nm and 1 um), respectively, when in suspension.</td>
<td>Solution in ultrapure water (Rg/L) Agitation Exposure dose DLS optical microscopy. Prolonged assay 8 days. Concentrations tested: 0.1, 1, 10 and 20 ppm.</td>
<td>INA</td>
<td>Acute assay: 48h, USEPA 2024. No food given during the test. Groups: 1) control, 2) THF group, 3) filtered TiO$_2$, 5, 6, 2, 1, 2, 5, 6, 8, 10 ppm).</td>
<td>There was no concentration-effect curve so the EC$<em>{10}$/EC$</em>{50}$ could not be determined for any group. Pre-illumination increased the toxicity of the two nano-TiO$_2$ products. E.g.: at 1 and 3.3 mg/L of product 1, immobilization went from 0 to 20% and from 28 to 73%, respectively, when there was pre-illumination.</td>
</tr>
<tr>
<td>D. magna</td>
<td>Product 1: 23nn, mainly anatase. Product 2: 180nm, 100% rutile.</td>
<td>Solutions were prepared in three ways: 1) Dilution in distilled water? sonication for 30 min. 2) 20 mg were placed in 300 mL THF? pulsed with nitrogen for 30 minutes and returned to distilled water? evaporation of the THF? $1$ min. 3) Same as 2, but without THF.</td>
<td>TEM: Characterization According to Levern and Klaper.</td>
<td>Acute assay: 48h, USEPA 2024. No food given during the test. Groups: 1) control, 2) THF group, 3) filtered TiO$_2$, 5, 6, 2, 1, 2, 5, 6, 8, 10 ppm, and 4) sonicated and non-filtered TiO$_2$ (50, 200, 250, 300, 400, and 500 ppm).</td>
<td>Filtered TiO$<em>2$: there was no mortality at 0.2 ppm, but 1% mortality at 1 ppm, LC$</em>{50}$: 0.5 ppm, NOEC: 2 ppm, EC$<em>{10}$/EC$</em>{50}$: 1 ppm. Sonicated TiO$<em>2$: no group suffered mortality &gt; 9%. NOEC, LOEC and LC$</em>{50}$ not applicable.</td>
</tr>
</tbody>
</table>

BCF = bioconcentration factor  
BET = Brunauer, Emmett, Teller method for surface area calculation  
CAT = catalase activity  
DLS = dynamic light scattering  
INA = information not available  
EC$_{10}$ = effective concentration for 10% of exposed organisms  
EC$_{50}$ = effective concentration for 50% of exposed organisms  
GPX = glutathione peroxidase activity  
GST = glutathione S-transferase activity  
ICP-OES = inductively coupled plasma optical emission spectrophotometry  
IP = effective concentration for 50% of exposed organisms  
LOEC = lowest observed effect concentration  
NOEC = no observed effect concentration  
ZP = zeta potential  
SEM = scanning electron microscopy  
SOD = superoxide dismutase activity  
TEM = transmission electron microscopy  
THF = tetrahydrofuran
### Table 2. Summary of papers published about the effects of nano-TiO\textsubscript{2} used in toxicology studies on fishes (Continues)

<table>
<thead>
<tr>
<th>Test species</th>
<th>Product used</th>
<th>Treatment of the product</th>
<th>Physicochemical characterization</th>
<th>Bioassay</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. rerio</em> adults (Xiong et al., 2011)</td>
<td>nano-TiO\textsubscript{2} from Nanjing University of Technology (anatase, purity 99%, diameter 20-70 nm, hydrodynamic diameter 25-63 nm, ZP -13±1 mV) bulk TiO\textsubscript{2} from Tianjin Guangcheng Chemical Reagent Co. (anatase, purity 99%, diameter 123-649 nm, hydrodynamic diameter 373-597, ZP -27.8±6 mV)</td>
<td>test suspension in aerated single distilled water? sonication (1.5 L, 100 W, 40 kHz for 20 min).</td>
<td>TEM, DLS</td>
<td>Acute assay 96h, semi-static (solution changed every 24h). No food given during the test. Concentrations tested: 0, 10, 50, 100, 150, 200 and 300 mg/L. From biomarkers and as, fish were exposed to 50 mg/L under light or dark conditions.</td>
<td>nano-TiO\textsubscript{2}, L50 = 124.5 mg/L. SOD activity decreased in liver tissues and increased in gut tissues, in both groups (under light or dark condition). CAT activity in liver tissue was observed to be induced in both groups. There was elevated protein carbonyl levels. Lipid peroxides were also found in the gill and gut tissues. GSH content (increased in gut tissue, and under dark condition) decreased in liver. MDA concentrations increased in gills and gut tissues. Morphological changes in gill include: (1) membrane damage, irregular cell outlines, pyknotic nuclei and a trend of complete disruption of cell cells). bulk TiO\textsubscript{2}, L50 &gt; 300 mg/L. No changes in SOD and CAT activities and in MDA content. There was an increase in GSH in gut tissue.</td>
</tr>
<tr>
<td><em>O. mykiss</em> (Bhattacharyya et al., 2010)</td>
<td>Nano-TiO\textsubscript{2} (54.2±73 nm, ZP -9), bulk TiO\textsubscript{2} and ionic titanium (titanium metal standard solution, FisherScient.k)</td>
<td>Stock solution (250 mg/L) in ultrapure water? sonication (30 min)? exposure dosage.</td>
<td>TEM, ICP-MS, DLS, particle size, CARS, multiphoton microscopy.</td>
<td>Prolonged assay 10 days, semi-static (change of 50% of the water every 2 days). Concentrations tested: 500 ppm of nano-TiO\textsubscript{2} and 5000 ppm of bulk TiO\textsubscript{2} and ionic Ti. Test exposure via diet. Test concentrations tested: 0.01 and 0.1 ppm TiO\textsubscript{2} in food.</td>
<td>No significant absorption of Ti was detected in any group. The Ti concentration in the gills increased in the group exposed to ionic Ti. High levels of Ti were found in the stomach of fish fed with medium and high doses of TiO\textsubscript{2}. TiO\textsubscript{2} aggregates were found on the surface of the gill epithelium after 20 and 96 h of exposure and made lamelle a after 14 days of exposure.</td>
</tr>
<tr>
<td><em>D. rerio</em> adult (Palaniappan et al., 2010)</td>
<td>Sigma Aldrich nano-TiO\textsubscript{2} (purity 99.7%, anatase, 20 nm, 200±50 µg/g). Particle size: 14 ±1, 5±nm. Nano-Chemicals bulk TiO\textsubscript{2} (99.7% purity, anatase).</td>
<td>Stock solution (10 ppm in ultrapure water? sonication (6 h)? storage at -20°C? sonication (30 min)? exposure dosage.</td>
<td>TEM.</td>
<td>Prolonged assay 14 days. Concentrations tested: 10 ppm of nano-TiO\textsubscript{2} or 100 ppm of bulk TiO\textsubscript{2}.</td>
<td>Mortality was not observed during the experiment. The biochemical constituents of the gill showed alterations. These alterations were greater in the group exposed to nano-TiO\textsubscript{2} than one exposed to bulk TiO\textsubscript{2}. Example: alterations in the amino bands.</td>
</tr>
<tr>
<td><em>D. rerio</em> (Zhu et al., 2010a)</td>
<td>Degussa P25 nano TiO\textsubscript{2}, (21 nm).</td>
<td>Stock solution (1 g/L in ultrapure water? sonication (10 min, 500 W, 400 kHz)</td>
<td>SEM, DLS.</td>
<td>Trophic transfer test. Daphnids were exposed to 0.1 or 1 mg/L of TiO\textsubscript{2} for 24h, after which they were added to in vivo culture medium and supplied to <em>D. rerio</em> as food. The test involved 14 days of absorption followed by 7 days of depuration (feeding with non-aminated daphnids). The TiO\textsubscript{2} concentration in the daphnids was determined as follows: 4.52 ± 0.36 mg/g (in the group exposed to 0.1 mg/L) and 61.09 ± 3.24 mg/g (in the group exposed to 1 mg/L). The fish were sampled on days 0, 1, 3, 5, 7, 10, 14, 15, 17, 19 and 21.</td>
<td>Prolonged exposure test. Concentration of Ti in the fish group fed with daphnids 0.1 mg/L = 106.3 ± 14.89 mg/kg and group fed with daphnids 1 mg/L = 52.02 ± 12.94 mg/kg.</td>
</tr>
</tbody>
</table>
Table 2. Summary of papers published about the effects of nano-TiO$_2$ used in toxicology studies on fishes (Continues)

<table>
<thead>
<tr>
<th>Study</th>
<th>Material used</th>
<th>Assay</th>
<th>Concentration Tested</th>
<th>Significance</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. rerio</em> adult vs juveniles (Griffith et al., 2009)</td>
<td>Degussa P25 nano-TiO$_2$ (45.4nm, 25nm rutile)</td>
<td>Acute assay: 48h static. Exposure dosage.</td>
<td>1000µg/L</td>
<td>Significant difference in the expression of 171 genes (microarray). No up-regulated or down-regulated genes were affected by exposure to nano-copper and nano-silver.</td>
<td>No mortality occurred, but a 1% increase in LPO and decrease in SOD, CAT, and POD activity. The liver was more sensitive than the gills and brain. Histopathological alterations were observed mainly at the highest concentrations. The liver showed vacuolization of cytoplasm and autolysosomes, including nuclear cell bodies and nuclear fragments that looked like apoptotic bodies and some loss of lipidosis. The gills showed thickening, edema, fusion, and hyperplasia of the lamellae and filaments.</td>
</tr>
<tr>
<td><em>C. carpio</em> juvenile vs control (Hao et al., 2009)</td>
<td>Sigma Aldrich nano-TiO$_2$ (32.4nm, 46.3nm, purity &gt;99.9%, rutile and anatase)</td>
<td>Chronic assay: 8 days semi-static. Solution changed daily. Animals were collected on days 2, 5, 10, 15, 20, and 25. Fish was given once a day during the test. Exposure dosage.</td>
<td>50, 100, 200mg/L</td>
<td>No significant difference found in blood IBARS at any time compared with the control. The histopathological analysis showed an alteration in the kidneys, but the TEM showed small aggregates apparently encapsulated around the tubules. Concentrations fluctuated in both the controls and the injected animals, but no effect was found in the plasma protein concentration.</td>
<td></td>
</tr>
<tr>
<td><em>C. carpio</em> juvenile vs control (Saras et al., 2009)</td>
<td>Sigma Aldrich nano-TiO$_2$ (32.4nm, 46.3nm, purity &gt;99.9%, rutile and anatase)</td>
<td>Chronic assay: 8 days semi-static. Solution changed daily. Solution (1mg/mL) in ringer. Exposure dosage.</td>
<td>1mg/mL</td>
<td>No significant difference in the concentration of As in the kidneys did not change significantly from 6h to 21 days post-injection, but after 90 days the concentration in the kidneys was significantly lower. The Ti level in the liver was approximately 15-fold lower.</td>
<td>No significant difference in the concentration of As in the kidneys did not change significantly from 6h to 21 days post-injection, but after 90 days the concentration in the kidneys was significantly lower. The Ti level in the liver was approximately 15-fold lower.</td>
</tr>
<tr>
<td><em>C. carpio</em> adult vs juvenile (Griffith et al., 2009)</td>
<td>Degussa P25 nano-TiO$_2$ (50nm, 25nm)</td>
<td>Acute assay: 48h static. Concentration tested: 300µg/mL.</td>
<td>50&gt;10mg/L</td>
<td>No mortality occurred, but a 1% increase in LPO and decrease in SOD, CAT, and POD activity. The liver was more sensitive than the gills and brain. Histopathological alterations were observed mainly at the highest concentrations. The liver showed vacuolization of cytoplasm and autolysosomes, including nuclear cell bodies and nuclear fragments that looked like apoptotic bodies and some loss of lipidosis. The gills showed thickening, edema, fusion, and hyperplasia of the lamellae and filaments.</td>
<td></td>
</tr>
<tr>
<td><em>D. rerio</em> adult vs juvenile (Griffith et al., 2009)</td>
<td>Degussa P25 nano-TiO$_2$ (30% rutile, 50% anatase)</td>
<td>Acute assay: 48h static. Concentration tested: 300µg/mL.</td>
<td>50&gt;10mg/L</td>
<td>No mortality occurred, but a 1% increase in LPO and decrease in SOD, CAT, and POD activity. The liver was more sensitive than the gills and brain. Histopathological alterations were observed mainly at the highest concentrations. The liver showed vacuolization of cytoplasm and autolysosomes, including nuclear cell bodies and nuclear fragments that looked like apoptotic bodies and some loss of lipidosis. The gills showed thickening, edema, fusion, and hyperplasia of the lamellae and filaments.</td>
<td></td>
</tr>
</tbody>
</table>

**Footnotes:**
- BET, CoBaller LS 13 320; polydispersion 0.197, largest particle diameter observed in suspension = 687.5nm.
- LC, 80% anatase, 45.4nm rutile.
- IC$_{50}$ >10mg/L of nanoparticles.
### Table 2. Summary of papers published about the effects of nano-TiO\textsubscript{2} used in toxicology studies on fishes

<table>
<thead>
<tr>
<th>Species</th>
<th>Stock solution</th>
<th>Storage</th>
<th>Assay type</th>
<th>LC\textsubscript{50}</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. mykiss</em></td>
<td>Deguza P25 nano-TiO\textsubscript{2} (23nm, 30±15mg/g, 75% rutile, 25% anatase, purity 99.5%)</td>
<td>Storage: sonication (6h, 35kHz)</td>
<td>TEM, spectral scans.</td>
<td>Prolonged away 4 days semi-static (80% of the water changed every 12h) Concentrations tested: 0.1, 0.5 and 1 mg/L.</td>
<td>There was no mortality. The fish did not accumulate Ti. Nitrite accumulation was observed at the highest concentration.</td>
</tr>
<tr>
<td><em>C. carpio</em></td>
<td>Deguza P25 nano-TiO\textsubscript{2} (50mg/ml, 21nm).</td>
<td>Storage: sonication (6h, 35kHz)</td>
<td>Laser particle analyzer, zeta potential analyzer</td>
<td>Chronic assay. Adsorption of Cd on TiO\textsubscript{2} and natural limatic particles (SP) were evaluated. Cd was added to the water (97.3 ± 6.9µg/L). Fish, followed by TiO\textsubscript{2} (0mg/L) or SP (10mg/L). The animals were placed in the water at 2 hours after food was given twice a day during the test.</td>
<td>TiO\textsubscript{2} showed higher capacity to adsorb Cd than SP. SP did not have a significant influence on Cd in fish. The presence of TiO\textsubscript{2} elevated the accumulation of Cd. After 25 days of exposure, the concentration of Cd increased by 146%, and 22µg/g. There was a positive correlation between the concentration of TiO\textsubscript{2} and Cd. TiO\textsubscript{2} and Cd accumulated mainly in the viscera and gills.</td>
</tr>
<tr>
<td><em>G. melita juveniles</em> (Warbelti et al., 2007b)</td>
<td>DuPont Haskell, Fine TiO\textsubscript{2} (930mm in water, 5.6g/100g, 99% rutile, 99% TiO\textsubscript{2}, and 1% aluminium)</td>
<td>Storage: sonication (10min, 50W/l, 40kHz)</td>
<td>TEM, X-ray fluorescence, X-ray diffraction</td>
<td>Acute assay 96h static. OECD 203. Concentrations tested: 0.1, 1, 10 and 100mg/L.</td>
<td>LC\textsubscript{50} 96h = 300mg/L for both types of TiO\textsubscript{2}. There was 10% of immobility at the concentrations of 10 and 100mg/L at end of 96h in both groups exposed to fine TiO\textsubscript{2}.</td>
</tr>
</tbody>
</table>

** Abbreviations:**
- BCF = bioconcentration factor
- BET = Brunauer, Emmett, Teller method for surface area calculation
- CARS = coherent anti-Stokes Raman scattering
- CAT = catalase activity
- DLS = dynamic light scattering
- ICP-MS = inductively coupled plasma mass spectroscopy
- LC\textsubscript{50} = lethal concentration for 50% of exposed organisms
- LPO = lipid peroxidation
- NOEC = no observed effect concentration
- POD = peroxidase
- SEM = scanning electron microscopy
- SOD = superoxide dismutase activity
- TBARS = thiobarbituric acid reactive substance assay
- TEM = transmission electron microscopy
- THF = tetrahydrofuran
- ZP = zeta potential

---

**Sources:**
- Federici et al., 2007
- Guemes et al., 2007
- Warbelti et al., 2007b

**Notes:**
- TEM = transmission electron microscopy
- ICP-MS = inductively coupled plasma mass spectroscopy
- LC\textsubscript{50} = lethal concentration for 50% of exposed organisms
- LPO = lipid peroxidation
- NOEC = no observed effect concentration
- POD = peroxidase
- ZP = zeta potential
<table>
<thead>
<tr>
<th>Test species</th>
<th>Product tested</th>
<th>Treatment of the product</th>
<th>Physicochemical characterization</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>polychaete, Arenicola marina (Galloway et al., 2010)</td>
<td>Sigma-Aldrich nano-TiO₂ cat. no. 634662-1 (23.2 nm, equivalent spherical diameter 32.4 nm, 46.3 m²/g, 99.9%, mixture of anatase and rutile; K 82.3 ppm, Zn 9.7 ppm, Na 6.0 ppm, Fe 3.1 ppm, Li 0.4 ppm).</td>
<td>Stock solution in ultrapure water? sonication (30 min)? mixed with natural treated sediment (collected at the same site where the animals were collected).</td>
<td>TEM, X-ray diffraction, ICP-OES</td>
<td>Prolonged assay 10 days. OECD/ASTM 1990. Exposure in seawater. Semi-static test (water changed every 48h). Feeding during the test. Concentrations tested: 1 to 3 g/kg of sediment. The organic content of the sediment was 0.33±0.4%. No behavioral alterations were detected. A change was observed in the feeding rate of the group exposed to 2 g/kg of nano-TiO₂ but not in the group exposed to 1 g/kg. No effect of exposure time was found. At 2 and 3 g/kg of nano-TiO₂ an impact was detected in the liposome stability (neutral red retention) and an increase in genetic impairment (comet assay). Bulk TiO₂ did not alter the rate of genetic damage compared to the control. Microscopy revealed TiO₂ aggregates of &gt;200 nm surrounding intestinal microvilli, but no absorption by the intestinal epithelium, although TiO₂ remained in the lumen. BCF = 0.156±0.075 (group 1 g/kg) and 0.196±0.038 (group 3 g/kg).</td>
</tr>
<tr>
<td>mollusk, Mytilus galloprovincialis (Canesi et al., 2010)</td>
<td>Degussa P25 nano-TiO₂ (purity &gt;99.5%)</td>
<td>Stock suspension (100µg/ml) in artificial seawater? sonication (5min, 100W, in a cold bath)? storage? sonication? exposure dosage</td>
<td>TEM, BET, DLS</td>
<td>Acute assay 24h. No feeding during the test. Concentrations tested: 0.05; 0.2; 1; 5 mg/L. No mortality was found in any exposure condition. There was destabilization of lysosomal membrane in hemocytes at 1 and 5 mg/L and in the digestive glands at 0.2, 1 and 5 mg/L. As well as accumulation of lipofuscin and lysosomal neutral lipids in the digestive glands at 1 and 5 mg/L, and an increase in CAT at 1 and 5 mg/L and in GST at 0.2, 1 and 5 mg/L in the digestive glands.</td>
</tr>
</tbody>
</table>

BCF = bioconcentration factor
BET= Brunauer, Emmett, Teller method for surface area calculation
CAT = catalase activity
DLS = dynamic light scattering
GST = glutathione S-transferase activity
TEM = transmission electron microscopy
concentration in supernatant after 16 hours went from 100 to 83 mg/L in bidistilled water and to 33 mg/L in surface water, while agglomeration and sedimentation of coated TiO$_2$ were slow. Some studies have involved semi-static aquatic bioassays, changing the exposure medium every 24-48 hours (Tables 1, 2 and 3), while others have performed static assays involving mainly acute exposure.

The literature reports nano-TiO$_2$ aggregates of about 500 nm in water, but this number varies greatly as a function of products and treatments used. Most aquatic tests have been performed starting from the sonication of a stock solution, while few have involved only agitation or filtration of the solution (Tables 1, 2 and 3). Adams et al. (2006) employed only agitation of Sigma Aldrich nano-TiO$_2$ in water and observed that 65nm sized particles formed aggregates of 320 nm, while larger particles of 950 nm and 44 μm formed aggregates of 320 nm and 1 μm, respectively. Zhu et al. (2010b) report that in a culture medium for daphnids, even with sonication, P25 formed aggregates that increased over time: 580 nm (1h), 2349 nm (12h) and 3528.6 nm (24h). The aggregation state of NPs inevitably changes with dilution, but there is a growing discussion about the use of dispersants or sonication processes to increase the dispersion of NPs in suspension in aquatic toxicology studies, in view of their environmental applicability (Baveye and Laba, 2008, Crane et al., 2008). One argument is that the study of non-dispersive materials would be of greater relevance to what actually takes place in the environment. Moreover, sonication may cause structural changes in nanomaterials, in fact, when performed in natural waters or in the presence of any electron donor, it may result in the generation of reactive oxygen species. The sonication time required changes according to the total concentration of the nanomaterial, and once sonication or agitation has stopped, the material does not remain dispersed for very long. On the other hand, the existence of natural dispersants in the environment, such as organic matter, would validate such studies (Crane et al., 2008). However, one should not assume that aggregate materials will necessarily not be bioavailable. They may simply change the mode of respiratory exposure on the water column to exposure via diet through sediment (Handy et al., 2008). Benthic organisms may be more exposed to NPs aggregates than to the material in the liquid phase. Similarly, the high concentration of ions in hard or marine waters will tend to cause aggregation of NPs, modifying the mode of exposure or organisms in these ecosystems (Handy et al., 2008).

A large part of acute exposure studies have been performed by withholding food from animals on the day prior to and during the bioassay. In the case of prolonged exposure, daily feeding has generally been maintained, with a few exceptions (Federici et al., 2007, Hao et al., 2009). However, it should be noted that this is also a point to be evaluated carefully and standardized, in view of the capacity of organic matter to adsorb TiO$_2$.

The diversity of manufactured TiO$_2$ NPs, the quality of the medium, the aquatic species tested, and the objectives of each research, require that exposure conditions be evaluated separately.

CONCLUSION

Evaluating the potential biological impact of nanomaterials has become increasingly important in recent years. This is particularly relevant because the rapid pace of nanotechnology development has not been accompanied by a complete investigation of its safety or by the development of suitable methodologies for this investigation.

Concern about the environmental consequences of nanotechnology has been growing and has reached public opinion. Nano-TiO$_2$ is a nanoproduct with applications in a variety of areas, and is also promising for the remediation of contaminated environments. However, its potentially harmful effects should be investigated in depth to ensure its sustainable use. Because water bodies are the final destination of contaminants, the evaluation of the effects of nano-TiO$_2$ on aquatic organisms is extremely necessary. Several groups have started research in this area, however, their results are still not conclusive and the need remains to continue researching. In fact, the results vary considerably, probably due to differences in the experimental models and products tested. Therefore, we agree with the recommendation that nanoecotoxicology studies focus on the characterization of NPs and that the best exposure conditions for the different NPs be analyzed (considering their particular properties), in the attempt to standardize bioassays and facilitate the comparison of results. In addition, the standardization of nanoecotoxicological methodologies is useful for the construction of protocols to underpin and guide public policies.

ACKNOWLEDGMENTS

The authors thank CAPES and Rede Nanobiotec for awarding a doctoral grant to Zaira Clemente, as well as the Brazilian research funding agencies FAPESP, CNPq and FUNDUNESP for their financial support of this work.
REFERENCES


Federici, G., Shaw, B. J. and Handy, R. D. (2007). Toxicity of titanium dioxide nanoparticles to rainbow trout (Oncorhyncus mykiss): gill injury, oxidative stress, and other physiological effects. Aquatic Toxicology, 84 (4), 415-430.


American Journal of Respiratory Cell and Molecular Biology, 6, 535-542.


