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Ecotoxicology of Nano-TiO_2 – An Evaluation of its Toxicity to Organisms of Aquatic Ecosystems

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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 (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

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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, TiO_2 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). TiO_2 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).

TiO_2 is obtained mainly from ore containing ilmenite ($FeTiO_2$), natural rutile (TiO_2) and leucoxene-like ilmenite. TiO_2 particles are referred to as primary, aggregates or agglomerates. Primary particles are individual crystals bound by crystal planes. Aggregates 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 TiO_2 are given in Diebold, 2003). Several TiO_2 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 TiO_2 and nano-TiO_2 is the larger surface area of a given mass or volume of NPs compared to an equivalent mass or volume of bulk TiO_2 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 TiO_2 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-TiO_2 have become popular due to their greater transparency on the skin compared to the white appearance of formulations containing bulk TiO_2.

The theoretical calculations of Palmer et al. (1990) and experimental data of Sakamoto et al. (1995) showed that the UVB attenuation of submicrometric TiO_2 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). TiO_2 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 (E_V), where the electrons cannot move freely, and the higher region is the conduction band (E_C), 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 (E_G) causes the promotion of an electron from the E_V to the E_C, with the concomitant generation of a gap (h+) in the E_V. 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. E_V 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•, O_2•, HO_2•, 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 E_G required for a photon to cause the photogeneration of charged species in TiO_2

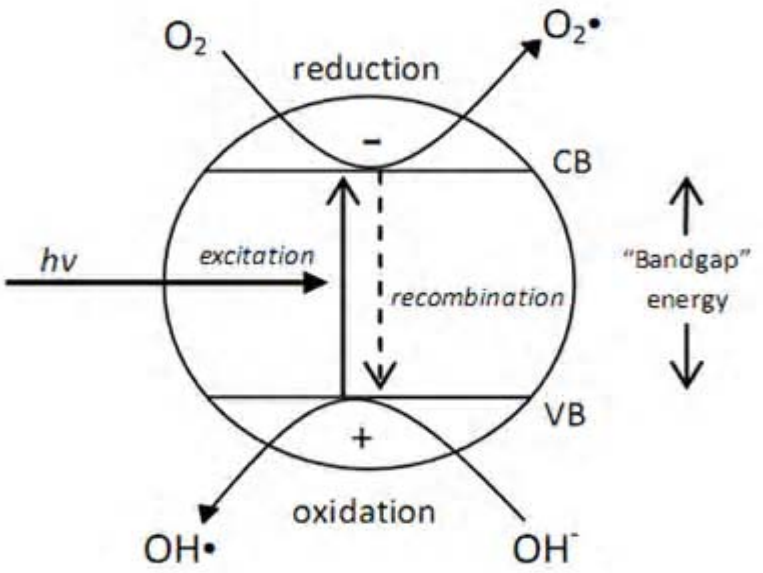

Fig. 1. Schematic representation of a TiO_2 particle, where E_V and E_C are the Valence Band and Conduction Band, respectively (adapted from Nogueira and Jardim, 1998)

(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_2O 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 towards 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., 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).

Nanoecotoxicology 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 nanoecotoxicological 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, Wiench *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) (Wiench *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 TiO_2 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-TiO_2 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-TiO_2 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-TiO_2 (Canesi *et al.*, 2010a, Galloway *et al.*, 2010). The intravenous administration of high doses of nano-TiO_2 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 TiO_2 was not complete (Zhu *et al.*, 2010b).

With regard to genotoxicity in aquatic organisms, nano-TiO_2 presents controversial results. Nano-TiO_2 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-TiO_2 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-TiO_2. The immune system also appears to be an important target of TiO_2 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 TiO_2 is exposed to UV radiation (Brezová *et al.*, 2005). Several studies have reported the phototoxic effects of TiO_2 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-TiO_2 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-TiO_2 and ultraviolet radiation increases cyto- and genotoxicity in fish cells (Reeves *et al.*, 2008, Vevers and Jha, 2008). The pre- and co-illumination of nano-TiO_2 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 nanoecotoxicological 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-TiO_2 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 CL_{50} up to the concentration of 100mg/L, however, nano-TiO_2 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 TiO_2 does not disperse well at 10-100 mg/L and that sedimentation occurs within 24-48 hours. For uncoated TiO_2 (BASF, >99%, 70% anatase, 30% rutile, 20-30nm, 48.6m^2/g), the

Table 1. Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on microcrustaceans (Continues)

Test species	*Product tested*	*Treatment of the product*	*Physicochemical characterization*	*Bioassay*	*Results*
D. magna (Kim et al., 2010)	Sigma Aldrich nano-TiO_2 (40 nm, 30% rutile, 70% anatase)	10% solution in water with pH 2 (without sonication) ? stock solution (1mg/L) in moderately hard synthetic water (MHW).	N4 and DLS submicron particle analyzer.	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 were 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 TiO_2. GPX and GST were also tested after fractionation of the nanoparticles (<200, <400, and <800 nm)	Acute assay: mortality did not reach 50% even at 10mg/l, so the LC_{50} could not be determined. Chronic assay: highest mortality at 5 and 10 mg/l (70 and 80%, respectively). No reproductive impairment observed. Increase in CAT at 10mg/l, no difference in SOD, GPx highest at 5mg/l, GST increased at 5 and 10 mg/l. TiO_2 was found in the intestines of daphnids and glued to their antennae and external surface.
D. magna (Rosenkranz, 2010)	Degussa P25 nano-TiO_2	100mg/L solution was prepared in culture medium for daphnids? sonication (30 min). The remaining solutions were made from serial dilutions of 1:10.	INA	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	Acute assay 48h: 10% mortality at 100mg/L. High molt frequency, dose-dependent. Chronic assay: high molt frequency only on the first day of exposure, at 1mg/L.
D. magna (Zhu et al., 2010b)	Degussa P25 nano-TiO_2 (21nm, 50m^2/g, 20% rutile, 80% anatase) Size of aggregates in culture medium: 1h - 580.5 nm; 12h – 2349.0 nm 24h – 3528.6 nm	Stock solution (1g/l) in ultrapure water ? sonication (10 min, 50 W/L, 40kHz) ? new sonication (10 min, 50W/L, 40kHz) prior to dilution in culture medium for daphnids.	SEM, DLS ICP-OES (concentration of Ti in the solution and in daphnids).	Acute assay 72h semi-static. OECD 202. Medium renewed daily. No food during the test. Concentrations tested: 0.1, 0.5, 1, 5, 10, 50 and 100 mg/L. Chronic assay 21 days semi-static. OECD 211. Daily renewal of medium and daily feeding. Concentrations tested: 0.1, 0.5, 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.	Acute assay In 48h: NOEC <50mg/L, EC_{50} > 100mg/L, LC_{50} > 100mg/L. In 72 h: NOEC<0.1 mg/L ; EC_{50} = 1.62 mg/L; LC_{50} = 2.02 mg/L. Chronic assay At 0.1 mg/L reproduction declined. At 0.5mg/l reproduction and growth were inhibited. Mortality was recorded in groups 1 and 5 mg/L after 8 days of exposure. EC_{50} = 0.46mg/L, LC_{50} = 2.62mg/L. The feeding rate decreased as the exposure concentration increased (exposure of 5h). Bioaccumulation test Group 0.1 mg/L: Concentration plateau in 12 h, BCF= 5.66x 10^4, time elapsed to accumulate 50% of the saturation level = 3.87h, time to reach 50% depuration = 26.76h. Group 1mg/L: Plateau in 24h; BCF = 1.18x10^5, time elapsed to accumulate 50% of the saturation level = 3.72h, time to reach 50% depuration = 74.52h. Depuration was not complete, 20% of the saturation concentration remained in the daphnids at the end of the experiment. Feeding during exposure to TiO_2 increased the accumulation time and reduced the depuration time.

Table 1. Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on microcrustaceans (Continues)

D. magna and *Chironomus riparius* (larvæ) (Lee et al., 2009)	Sigma Aldrich nano-TiO_2 7nm (300.81m^2/g) and 20 nm (66.604m^2/g)	Solution (1mg/L) in culture medium ? sonication (15 min).	TEM, BET	Acute assay 96h. OECD 1984, 1998. Concentration tested: 1mg/l.	No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group.
D. magna (Strigul et al., 2009)	nano-TiO_2 prepared by hydrolysis of the titanium sulfate solution (6 nm, agglomerates 0.5 -2 mm)	Stock solution ? sonication (30min).	DLS	Acute assay 24 and 48h OECD 202. Concentrations tested: 2.5; 8; 25; 80; 250 mg/L.	TiO_2 presented low toxicity and LC_{50} could not be calculated. Animals exposed to 80 and 250mg/L for 24h were slower.
Daphnia magna (Wiench et al., 2009)	Bulk TiO_2 BASF nano-TiO_2: - non-coated (>99%; 70/30 anatase/rutile; 20-30nm; 48.6m^2/g) - T-LITE SF (80%; 50nm; 100m^2/g; rutile) - T-LITE SF-S - T-LITE SF-MAX It was found that 10-100 mg/L did not disperse well and sedimentation occurred in 24-48h. For non-coated TiO_2, after 16h in bidistilled water, the concentration in the supernatant went from 100 to 83mg/L and to 3.3 mg/L in surface water. For coated TiO_2, agglomeration and sedimentation were slow.	Stock solution (100mg/L) in demineralized water ? sonication (5min) or magnetic agitation (10 min) or both methods ? dispersion in M4 or SW medium (natural surface water) ? sonication and filtration (2um) ? UV irradiation (30 min 20W/m^2).	TEM, ultracentrifugation.	Acute assay 48h. OECD 202. Chronic assay 21 days. OECD 211. Only with T-Lite SF-S –semi-static assay (medium changed 3 times per week). Daily feeding. Concentrations tested: 0.01 a 100 mg/L.	Acute assay EC_{50}>100mg/L in at the treatments EC_{10} non-coated nano-TiO_2 sonicated in M4 = 85.1mg/L. In SW= 3.7mg/L. Bulk TiO_2 sonicated in M4= 91mg/L; in SW = 13.8mg/L. Chronic assay There was no mortality, but reproductive effects were observed. NOEC=3mg/L LOEC=10mg/L EC_{50}=26.6mg/L EC_{10} = 5.02mg/L.
D. pulex and *Ceriodaphnia dubia* (Griffith et al., 2008)	Degussa P25 nano-TiO_2 (20% rutile, 80% anatase, 45.41m^2/g; 20.5±6.7 nm; ZP -25, 1; polydisperse 0.197, largest particle diameter observed in suspension was 687.5nm)	Stock solution in ultrapure water (1mg/ml) ? sonication (6 W, 22.5 kHz, 6 one-half second pulses).	BET; Coulter LS 13 320; polydispersity; Zeta reader Mk 21-II; scanning micrographs.	Acute assay 48h static. American Society for testing and materials guidelines. No food given during the test.	LC_{50} >10mg/L for both tested organisms.
D. magna and *Thamnocephalus platyurus* (Heinlaan et al., 2008)	Sigma Aldrich nano-TiO_2 (25-70nm). Riedel-de Haen bulk TiO_2	Stock solution in ultrapure water (40g/L) ? sonication (30 min) ? storage at 4°C ? vortex ? exposure dosage.	INA	*D. magna*: Acute assay 48h, in the dark. Standard Operational Procedures of Daphtoxkit FTM magna (1996). *T. platyurus* (larvae): Acute assay 24h, in the dark. Thamnotoxkit FTM (1995). Concentrations tested: 0.01 to 20000 mg/L	*D. magna*: LC_{50} of nano-TiO_2 ~ 20000mg/L. (Bulk TiO_2 and other values of toxicity were not tested). *T. platyurus*: LC_{50}, LC_{20} and NOEC of nano- and bulk TiO_2 > 20.000 mg/L.

Table 1. Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on microcrustaceans

D. magna (Lovern et al., 2007)	Nano-TiO_2 30nm (in suspension)	THF was used to ensure dispersion. The THF was eliminated by evaporation and filtration and confirmed by spectrophotometry.	TEM. Characterization according to Lovern and Klaper (2006).	Acute assay 60 min. USEPA 23. Concentration tested: 2ppm (LOEC calculated in a previous experiment).	TiO_2 did not significantly alter the heat rate, jump, movement of appendices, and curvature of the abdominal claw.
D. magna (Warheit et al., 2007b)	DuPont Haskell TiO_2: fine TiO_2 (380nm in water, 5.8m^2/g, 100% rutile, 99% TiO_2 and 1% aluminum) afC (140 ± 44nm in water; 38..5m^2/g; 79% rutile 21% anatase; 90% TiO_2 ; 1% amorphous silica; 7% aluminum).	INA	DLS, BET, X-ray fluorescence, X-ray diffraction.	Acute assay 48h, static. OECD 202. Concentrations tested: 0.1, 1, 10 and 100 mg/L.	LC_{50} 48h >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.
D. magna (Adams et al., 2006)	Sigma Aldrich nano-TiO_2 65 nm, 950 nm and 44 µm. Smaller particles (65 nm) appeared larger (on average 320nm) and larger ones (950nm and 44 um) appeared smaller (320nm and 1um), respectively, when in suspension.	Solution in ultrapure water (10g/L) ? agitation ? exposure dosage	DLS optical microscopy.	Prolonged assay 8 days. Concentrations tested: 1, 10 and 20 ppm.	20 ppm of nano-TiO_2 was lethal for 40% of the organisms.
D. magna (Hund Rinke and Simon, 2006)	Product 1: 25nm, mainly anatase. Product 2: 100nm, 100% anatase.	The TiO_2 suspension was agitated and pre-illuminated in SUNTEST CPS. Particles were washed following the manufacturer's instructions ? mother solution ? sonication ? continuous agitation and irradiation in a solar light simulation system (300-800 nm 250W, 30 min) ? samples were transferred and incubated for 72h with visible light.	INA	Acute assay 48h. ISSO 6341, OECD 202 and DIN 38412-30. Concentrations tested: 1, 1.5, 2, 2.5, 3 mg/L.	There was no concentration-effect curve, so the EC_{50} could not be determined for any group. Pre-illumination increased the toxicity of the two nano-TiO_2 products. E.g.: at 1and 2.5 mg/L of product 1, immobilization went from 0 to 20% and from 28 to 73%, respectively, when there was pre-illumination.
D. magna (Lovern and Kapler, 2006)	INA. Mean diameter of filtered TiO_2: 30nm; in sonicated solution: 100 to 500 nm	Solutions were prepared in three ways: 1) Dilution in distilled water ? sonication for 30 min. 2) 20mg were placed in 200 ml THF ? pulverization with nitrogen ? over-night on moving plate ? filtration ? dilution in deionized water ? evaporation of the THF ? filtration. 3) Same as 2, but without THF.	TEM spectrophotometry.	Acute assay 48h. USEPA 2024. No food given during the test. Groups: 1) control, 2) THF group, 3) filtered TiO_2 (0.2, 1, 2, 5, 6, 8, and 10 ppm), and 4) sonicated and non-filtered TiO_2 (50, 200, 250, 300, 400, and 500 ppm).	Filtered TiO_2: there was no mortality at 0.2 ppm, but 1% mortality at 1ppm. LC50=5.5ppm; LOEC= 2 ppm; NOEC=1 ppm. Sonicated TiO_2: no group suffered mortality > 9%. NOEC, LOEC and LC_{50} not applicable. When there was no mortality, no immobility or swimming abnormalities were observed in any group.

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 spectroscopy
LC_{50} = lethal 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_2 used in toxicology studies on fishes (Continues)

Test species	*Product tested*	*Treatment of the product*	*Physicochemical characterization*	*Bioassay*	*Results*
D. rerio adults (Xiong et al., 2011)	nano-TiO_2 from Nanjing University of Technology (anatase, purity 99%, diameter 20-70nm, hydrodynamic diameter 251 – 630nm, ZP -13,1mV) bulk TiO_2 from Tianjin Guangcheng Chemical Reagent Co. (anatase, purity 99%, diameter 128-949nm, hydrodynamic diameter 272-597, ZP -27,8mV)	test suspension in aerated single-distilled water ? sonication (1,5 L, 100W, 40kHz for 20 min).	TEM, DLS	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 analysis, fish were exposed to 50mg/L under light or dark conditions.	nano-TiO_2 LC50 = 124,5 mg/L SOD activity decreased in liver tissues and increased in gut tissues, in both groups (under light or dark conditions). CAT activity in liver tissue was observed to be reduced in both groups. There was elevated protein carbonyl levels. Lipid peroxides were also found in the gills and gut tissues. GSH content increased in gut tissue, and (under dark conditions) decreased in liver. MDA concentrations increased in gills and gut tissues. Morphological changes in gill cells (cell membrane damage, irregular cell outlines, pyknotic nuclei and a trend of complete disruption of gill cells). bulk TiO_2 LC50 > 300mg/L no changes in SOD and CAT activities and in MDA content. There was an increase in GSH in gut tissue.
O. mykiss (Johnston et al., 2010)	Nano-TiO_2 (34.2±1,73nm, ZP -9), bulk TiO_2 and ionic titanium (titanium metal standard solution, Fisher Scientific).	Stock solution (250µg/L) in ultrapure water ? sonication (30min) ? exposure dosage.	TEM, ICP-MS, DLS, particle sizer, CARS, multiphoton microscopy.	Prolonged assay 10 days, semi-static (change of 50% of the water every 2 days). Concentrations tested: 500 (nano-TiO_2) and 5000 µg/L (nano- and bulk TiO_2 and ionic Ti). Test exposure via diet, 21 days. Concentrations tested: 0.01 and 0.1% nano-TiO_2 in food.	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_2. TiO_2 aggregates were found on the surface of the gill epithelium after 24 and 96h of exposure and inside lamellae after 14 days of exposure.
D. rerio adult (Palaniappan et al., 2010)	Sigma Aldrich nano-TiO_2 (purity 99.7%, anatase, 20nm, 200±20m^2/g). Particle size: 14.1±1.52nm. Nice Chemicals bulk TiO_2 (99.7% purity, anatase).	Stock solution (10 ppm) in ultrapure water ? sonication (6h) ? storage at -20°C ? sonication (30 min) ? exposure dosage.	TEM.	Prolonged assay 14 days. Concentrations tested: 10ppm of nano-TiO_2 or 100ppm of bulk TiO_2.	Mortality was not observed during the experiment. The biochemical constituents of the gills showed alterations. These alterations were greater in the group exposed to nano-TiO_2 than the one exposed to bulk TiO_2. Example: alterations in the amide I bands.
D. rerio (Zhu et al., 2010a)	Degussa P25 nano-TiO_2 (21nm).	Stock solution (1g/L in ultrapure water ? sonication (10 min, 50W/L, 40kHz).	SEM, DLS.	Trophic transfer test. Daphnids were exposed to 0.1 or 1mg/L of TiO_2 for 24h, after which they were collected and washed in culture medium and supplied to *D. rerio* as food. The test involved 14 days of absorption followed by 7 days of depuration (feeding with non-contaminated daphnids). The TiO_2 concentration in the daphnids was determined as follows: 4.52 ± 0.36 mg/g (in the group exposed to 0.1mg/L) and 61.09 ± 3.24 mg/g (in the group exposed to 1mg/L). The fish were sampled on days 0, 1, 3, 5, 7, 10, 14, 15, 17, 19 and 21. Prolonged exposure test. 14 days, followed by 7 days of depuration. Semi-static (water changed daily). Concentrations tested: 0.1 and 1mg/L.	No mortality or abnormalities were observed. Trophic transfer of TiO_2 occurred. There was no apparent biomagnification. Trophic transfer test Concentration of Ti in the fish group fed with daphnids 0.1 mg/l = 106.57 ± 14.89 mg/kg and group fed with daphnids 1 mg/l = 522.02 ± 12.94 mg/kg. BCF<1. Prolonged exposure test Fish accumulated TiO_2, reaching a plateau of about 1.5mg/kg on day 3 (group 0.1mg/L) and of 100 mg/kg on day 10 (group 1mg/L). BCF= 25.38 and 181.38 (at equilibrium for groups 0.1 and 1mg/L respectively). During the depuration phase, the concentration of TiO_2 in the entire body was found to decline.

Table 2. Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on fishes (Continues)

D. rerio female adults (Griffith et al., 2009)	Degussa P25 nano-TiO_2 (45.41m^2/g; ZP -25.1mV). Aggregates in powder 20.5±6.7nm; in suspension 220.8 to 687.5nm.	Stock solution in ultrapure water ? sonication (6s, 6W, 22kHz) ? exposure dosage.	BET, SEM, scanning micrographs.	Acute assay 48h static. Concentration tested: 1000µg/L.	Significant difference in the expression of 171 genes (microarray) - 60 up-regulated and 111 down-regulated (53 of these genes were affected by exposure to nano-copper and nano-silver). The affected genes were involved in ribosome structure and activity. No alteration was observed in genes related to regulation of oxidative stress. No histopathological differences were observed in the gills compared with the control group.
Cyprinus carpio juveniles (Hao et al., 2009)	Hongsheng Material nano-TiO_2 (50nm, 30±10m^2/g, rutile 98%).	Solution ? sonication (30min, 100W, 40kHz).	INA	Prolonged assay 8 days semi-static (solution changed daily). No food given during the test. Animals were collected on days 1, 2, 4 and 6 for biochemical analyses. For histopathology, the animals were exposed for 20 days. Concentrations tested: 10, 50, 100 and 200 mg/L.	No mortality occurred, but after 1h of exposure the respiratory rate and swimming rates increased, as well as the production of mucus, in a concentration-dependent way. The biomarkers of oxidative stress varied with the concentration and exposure time. At 100 and 200mg/L there was an 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 autosomes, including necrotic cell bodies and nuclear fragments that looked like apoptotic bodies and some foci of lipidosis. The gills showed thickening, edema, fusion and hyperplasia of the lamellae and filaments.
Oncorhynchus mykiss juveniles (Scown et al., 2009)	Sigma Aldrich nano-TiO_2 (32.4nm, 46.3m^2/g, purity >99.9%, anatase and rutile). Particle size: 34.2nm, 18.6m^2/g (in powder). 400-1100 nm (in ringer and water). ZP: 0 at -0.6mV.	Solution (100mg/L) in ringer ? sonication (30 min).	BET	Intravenous administration (1.3mg/kg). Fish and blood samples collected 6h and 90 days post-injection.	10 to 19% of injected Ti accumulated in the kidneys (up to 23µg/g). The concentration 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. Preliminary studies showed that Ti did not accumulate in the brain, gills or spleen. No significant difference was found in blood TBARS at any time compared with the control. The histopathological analysis showed no alteration in the kicheys, but the TEM showed small aggregates apparently encapsulated around the tubules. Creatinine levels fluctuated in both the controls and the injected animals, but no effect was found in the plasma protein concentration.
C. carpio (Sun et al., 2009)	Degussa P25 nano-TiO_2 (50m^2/g, 25nm) Arsenite (As III) prepared from As_2O_3.	Stock solution of nanoTiO_2 (1g/l) ? sonication (10min, 50W/l, 40kHz) ? exposure dosage.	INA	Chronic assay. Groups: 1) control, 2) only As III (200µg/L ± 10.2); 3) As III + TiO_2 (10mg/L ±1.3). Animals were placed in the aquariums 2h after the addition of As and TiO_2. Semi-static test (water changed daily). Animals were collected on days 2, 5, 10, 15, 20 and 25. Food was given once a day during the test. Speciation was evaluated of As in water, in the presence of TiO_2, with and without sunlight.	The concentration of As in the carps increased from 42% (20 days) to 185% (second day) in the presence of nano-TiO_2. The order of accumulation of As and TiO_2 in the different tissues was: viscera > gills > skin and scales > muscle. In the absence of sunlight, only a small amount of As III moved to As V (loaded, and therefore with less capacity to pass through biological membranes). With sunlight, about 75% of the As III moved to AsV in 1h.
Dario rerio adults and juveniles (Griffith et al., 2008)	Degussa P25 nano-TiO_2 (20% rutile, 80% anatase, 45.41m^2/g 20.5±6.7 nm; ZP -25.1; polydispersion 0.197, largest particle diameter observed in suspension = 687.5nm).	Stock solution (1mg/ml) in ultrapure water ? sonication (6W, 22.5kHz, 6 half-second pulses).	BET, Coulter LS 13 320; polydispersity; Zeta reader Mk 21-II; scanning micrographs.	Acute assay 48h static.	LC_{50}> 10mg/L of nanoparticles

Table 2. Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on fishes

O. mykiss juveniles (Federici et al., 2007)	Degussa P25 nano-TiO_2 (21nm, 50±15m^2/g, 75% rutile, 25% anatase, purity 99%). Particle sizes were close to those specified by the manufacturer (24.2±2.8nm). The concentration of TiO_2 (spectrometry) in the tank reached 95-98% of the target value 10 min after dosing. The concentration in water was measured before changing the solution, to confirm that the concentration remained unchanged in 12h.	Stock solution (10g/L) in ultrapure water ? sonication (6h, 35kHz) ? storage ? sonication (30 min) ? exposure dosage.	TEM, spectral scans.	Prolonged assay 14 days semi-static (80% of the water changed every 12h). Concentrations tested: 0.1; 0.5 and 1 mg/L. Food was withheld 24h prior to and during the test (except on day 10). Fish were sampled on days 7 and 14.	There was no mortality. The fish did not accumulate Ti. Changes in behavior and mucus secretion were observed at the highest concentration. The gills showed increased occurrence of edema in secondary lamellae, morphological changes in mucocyte, hyperplasia of primary lamellae, and aneurysm. Vacuolization and erosion of villosities in the intestines was observed, as well as loss of sinusoidals pace, some foci of lipidosis, occasional necrotic cells and apoptotic bodies in the liver. No changes were observed in the brain. There was no clear effect of the treatment or of time on the Ti levels in the gills, liver or muscle. No hematological change was found. There was alteration of the levels of tissue Zn and Cu. A concentration-dependent reduction was found in the Na-K ATPase activity in the gills, intestines and brain at the end of the experiment (significant differences only among some groups). In general, there was an increase in TBARS at the end of the experiment in gills, intestines and brain, but not in liver. Concentration-dependent glutathione depletion occurred only in liver on day 14.
C. carpio (Zhang et al., 2007)	Degussa P25 nano-TiO_2 (50m^2/g;21nm).	Stock solution in ultrapure water.	Laser particle analyzer, zeta potential analyzer, ICP-OES, atomic fluorescence spectroscopy.	Chronic assay. Adsorption of Cd on TiO_2 and natural sediment particles (SP) were evaluated. Cd was added to the water (97.3 ± 6.9µg/L) first, followed by TiO_2 (10mg/L) or SP (10mg/L). The animals were placed in the water 2 hours later. Food was given twice a day during the test. Fish were transferred to new solutions every day. The animals were sampled on days 2, 5, 10, 15, 20 and 25.	TiO_2 showed higher capacity to adsorb Cd than SP. SP did not have a significant influence on Cd in fish. The presence of TiO_2 elevated the accumulation of Cd. After 25 days of exposure, the concentration of Cd increased by 146%, and was 22µg/g. There was a positive correlation between the concentration of TiO_2 and Cd. TiO_2 and Cd accumulated mainly in the viscera and gills.
C. carpio (Sun et al., 2007)	Degussa P25 nano-TiO_2 (50m^2/g,25nm, aggregates of 50- 400nm in water.) Arsenate (As V) (prepared from Na_3AsO_4•$12H_2O$).	Stock solution of nano-TiO_2 (1g/l) ? sonication (10min, 50W/l, 40kHz) ? exposure dosage.	TEM	Chronic assay, semi-static (water changed daily). Groups: 1) control, 2) only As V (200ug/l± 10.2); 3) As V + TiO_2 (10mg/l ±1.3). Animals were placed in the aquariums 2h after the addition of As and TiO_2. Animals were collected on days 2, 5, 10, 15, 20 and 25. Food given once a day during the test.	
O. mykiss juveniles (Warheit et al., 2007b)	DuPont Haskell; Fine TiO_2 (380nm in water, 5.8m^2/g, 100% rutile, 99% TiO_2 and 1% aluminum). afiC (140±44 nm in water; 38.5m^2/g; 79% rutile, 21% anatase, 90% TiO_2; 1% amorphous silica; 7% aluminum).	INA	DLS, BET, X-ray fluorescence, X-ray diffraction.	Acute assay 96h static. OECD 203. Concentrations tested: 0.1, 1, 10 and 100 mg/L.	LC_{50} 96h>100mg/L for both types of TiO_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_2.

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-OES = inductively coupled plasma optical emission spectroscopy
ICP-MS = inductively coupled plasma mass spectroscopy
LC_{50} = lethal concentration for 50% of exposed organisms
LPO = lipid peroxidation
NOEC = no observed effect concentration
POD = peroxidase
ZP = zeta potential
SEM = scanning electron microscopy
SOD = superoxide dismutase activity
TBARS = thiobarbituric acid reactive substance assay
TEM = transmission electron *microscopy*
THF = tetrahydrofuran
INA = information not available

Table 3. Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on other aquatic organisms

Test species	*Product tested*	*Treatment of the product*	*Physicochemical characterization*	*Bioassay*	*Results*
polychaete *Arenicola marina* (Galloway et al., 2010)	Sigma-Aldrich nano-TiO_2 cat. no. 634662-1 (23.2 nm, equivalent spherical diameter 32.4 nm, 46.3 m^2/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). bulk TiO_2	Stock solution in ultrapure water ? sonication (30 min) ? mixed with natural treated sediment (collected at the same site where the animals were collected).	TEM, X-ray diffraction, ICP-OES	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-TiO_2 but not in the group exposed to 1g/kg. No effect of exposure time was found. At 2 and 3g/kg of nano-TiO_2, an impact was detected in the liposome stability (neutral red retention) and an increase in genetic impairment (comet assay). Bulk TiO_2 did not alter the rate of genetic damage compared to the control. Microscopy revealed TiO_2 aggregates of >200nm surrounding intestinal microvillosities, but no absorption by the intestinal epithelium, although TiO_2 remained in the lumen. BCF = 0.156± 0.075 (group 1g/kg) and 0.196±0.038 (group 3g/kg).
mollusk *Mytilus galloprovincialis* (Canesi et al., 2010)	Degussa P25 nano-TiO_2 (purity >99.5%)	Stock suspension (100µg/ml) in artificial seawater ? sonication (15min, 100W, in a cold bath) ? storage ? sonication ? exposure dosage.	TEM, BET, DLS.	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 the lysosomal membrane in hemocytes at 1 and 5mg/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.

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.

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