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Manufactured nanoparticles in the aquatic environment-biochemical responses on freshwater organisms: A critical overview

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ABSTRACT

The enormous investments in nanotechnology have led to an exponential increase of new manufactured nano-enabled materials whose impact in the aquatic systems is still largely unknown. Ecotoxicity and nanosafety studies mostly resulted in contradictory results and generally failed to clearly identify biological patterns that could be related specifically to nanotoxicity. Generation of reactive oxygen species (ROS) is one of the most discussed nanotoxicity mechanism in literature. ROS can induce oxidative stress (OS), resulting in cyto- and genotoxicity. The ROS overproduction can trigger the induction of anti-oxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidases (GPx), which are used as biomarkers of response. A critical overview of the biochemical responses induced by the presence of NPs on freshwater organisms is performed with a strong interest on indicators of ROS and general stress. A special focus will be given to the NPs transformations, including aggregation, and dissolution, in the exposure media and the produced biochemical endpoints.

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1. Introduction

Nanotechnology has emerged as a fast growing sector impacting key economical fields and providing new engineered nano-enabled products, constituted by nanoparticles (NPs), with novel and unique functions that reach the market every day (Bour et al., 2015). NPs are defined as materials with a size between 1 and 100 nm on at least one dimension, having unique physicochemical properties differing from their bulk forms due to their greater surface area to volume ratio. This size related-properties results in larger reactivity and higher mobility (Rauscher et al., 2014), leading to numerous applications in medical diagnostics, electronics, computers, cosmetics and environmental remediation. The worldwide consumption of NPs is expected to grow from 225,060 metric tons in 2014 to nearly 584,984 metric tons in 2019 representing an annual growth rate of 21.1% (Research, 2015). Although impressive, these numbers are in fact "expected" values obtained by estimation or modeling. The lack of legislation for nanotechnologies gives the manufacturers no onus to reveal the real figures, thus, indeed, these predicted values are most probably significantly higher. The absence of real numbers hinders the prediction of the NPs amount that are actually being released into the environment (Piccinno et al., 2012). Even though several studies have been performed with the goal of modeling NPs environmental concentrations (Gottschalk et al., 2013), they should only be considered as guidelines, since they derive from uncertain data about the NPs production (often obtained by surveys to the producers) and extrapolations used to scale up regional to worldwide amounts (Piccinno et al., 2012; Gottschalk et al., 2011; Keller et al., 2013).

When released in natural media NPs will be subjected to a dynamic physical and chemical environment that consequently results in different and unknown endpoints far from their pristine or as released state. Therefore, environments and humans are not facing pristine manufactured NPs but rather transformed nano-enabled products, which is factually accepted but so far neglected. In fact, the large majority of the physicochemical and toxicity data obtained so far was focused on simple nanoscale particles and not on relevant nano-enabled products. This includes not only the NP embedded in the manufactured matrix but also the materials resulting from the interaction with biotic and abiotic (bio) molecules composing the natural systems. To further complicate the interpretation of the NPs studies, there are two distinct mechanisms that should be considered but are not easily differentiated: (i) chemical toxicity by the release of possible ions and/or formation of reactive oxygen species (ROS) (Fu et al., 2014), and (ii) physical stress or stimuli caused by NPs size, shape and surface properties (Vale et al., 2014; Libralato et al., 2013). These materials are generally associated with cellular perturbations such as ROS generation, and gene expression and proteome profiles alterations. For these reasons, the NPs escalating production and applications has raised concerns about their environmental and human safety, which have led to large investments in nanosafety-related projects resulting in a considerable amount of data assessing their potential hazard (Savolainen et al., 2013). However, the establishment of relationships between bioavailable NP-containing species and the specific bioadverse or biocompatible endpoints is still lacking, mainly since, the effects are NP-dependent and also specie-dependent (Buric et al., 2015).

This work provides an overview of the latest studies on the impact of NPs onto freshwater ecosystems, considered by many as the ultimate sink of these particles, with a special focus on (i) NPs transformations and characterization in the different test media, and (ii) toxicological effects such as generation of ROS, genotoxicity, metallomic and proteomic changes. This survey is focused on metallic NPs including nAg, $nTiO_2$, nZnO and nCuO, mostly due to the great number of studies dedicated to these particles.

2. NPs transformations in aquatic systems

NPs can enter in an aquatic compartment from (i) wastewater treatment plants effluents, (ii) direct use (e.g., application of NPs-containing paintings on boats), and (iii) deposition from the air compartment. When entering aquatic compartment, NPs will be exposed to a highly dynamic physical and chemical environment that leads to several transformations that will change their pristine or as released physicochemical properties (Fig. 1). These transformations, including dissolution, aggregation and sedimentation, are dependent on both physicochemical properties of the NP (and nanoforms thereof) and those of the environment into which they were released.

Colloidal particles, including organic and inorganic matter, are ubiquitous in the aquatic environment and can be originated from both natural and anthropic sources. These colloids can strongly interact with NPs, thereby determining their forms over space and time (dynamic speciation), and greatly affecting their bioavailability. Thus, the NP will have a specific speciation in each environmental compartment, and this speciation is always dynamic with reaction rates that depend upon the chemical nature and physical sizes of the engineered and natural colloids. Although it is clear that dynamic speciation must be considered in order to make relevant predictions of NPs fate, toxicity and risk, until now this critical issue, was mostly neglected (see detailed explanation on Pinheiro and Domingos, 2015).

Dissolution, which is one of the main transformations of metallic NPs such as nZnO, nCuO, and nAg, is mainly due to (i) the formation of partially soluble metal-oxide (Heinlaan et al., 2008; Domingos et al., 2013a; Wang et al., 2015), (ii) the oxidation of the particle constituents (Ma et al., 2014; Wang et al., 2013a; Dale et al., 2013; Lok et al., 2007; Derfus et al., 2004), and (iii) the complexation of the particle constituent metal by complexants present in the environmental compartment or even in the NPs embedded matrix (including the manufactured stabilizers) (Domingos et al., 2013b; Domingos et al., 2014). The sulfidation of the metallic NPs can retard their oxidation and, thus, their dissolution (Ma et al., 2014; Wang et al., 2013a; Dale et al., 2013; Thalmann et al., 2014). This dissolution mechanism results in the release of toxic cations, such that their persistence is reduced but the toxicity is increased. Evidently, complete dissolution of the NPs allows the prediction of their impact using already existing models for metal speciation and toxicity.

Photoreactions can also be important transformations affecting the NPs coatings, oxidation state, generation of ROS, and persistence, which is the case of the innately photoactive TiO_2 and ZnO particles (Hund-Rinke and Simon, 2006; Zhang et al., 2007a).

Aggregation is other critical transformation, which mainly by interaction with naturally occurring bio- or geomacromolecules affect NPs size and surface chemistry. For example, organic matter (OM) provides both charge and steric stabilization (Mohammed et al., 2008; Domingos et al., 2009a) of the NPs, although they may also result in bridging flocculation when in presence of multiple charged cations and anions (Domingos et al., 2010). OM effects are complex and difficult to predict, however, is of extremely importance to explore these interactions since, OM concentrations are typically orders of magnitude higher in concentration than engineered NPs, and, thus, likely to substantially modify their properties and behaviors. Despite the significance of these interactions, with both organic and inorganic matter, and to the best of our knowledge, no relevant toxicity studies are available.

Dissolution and aggregation are dynamic processes that can decrease the NPs available surface area, thereby decreasing their reactivity. However, this decrease is dependent on the surface properties, particle number, size distribution, and the fractal dimensions of the aggregate (Hotze et al., 2010). The NP size will affect its

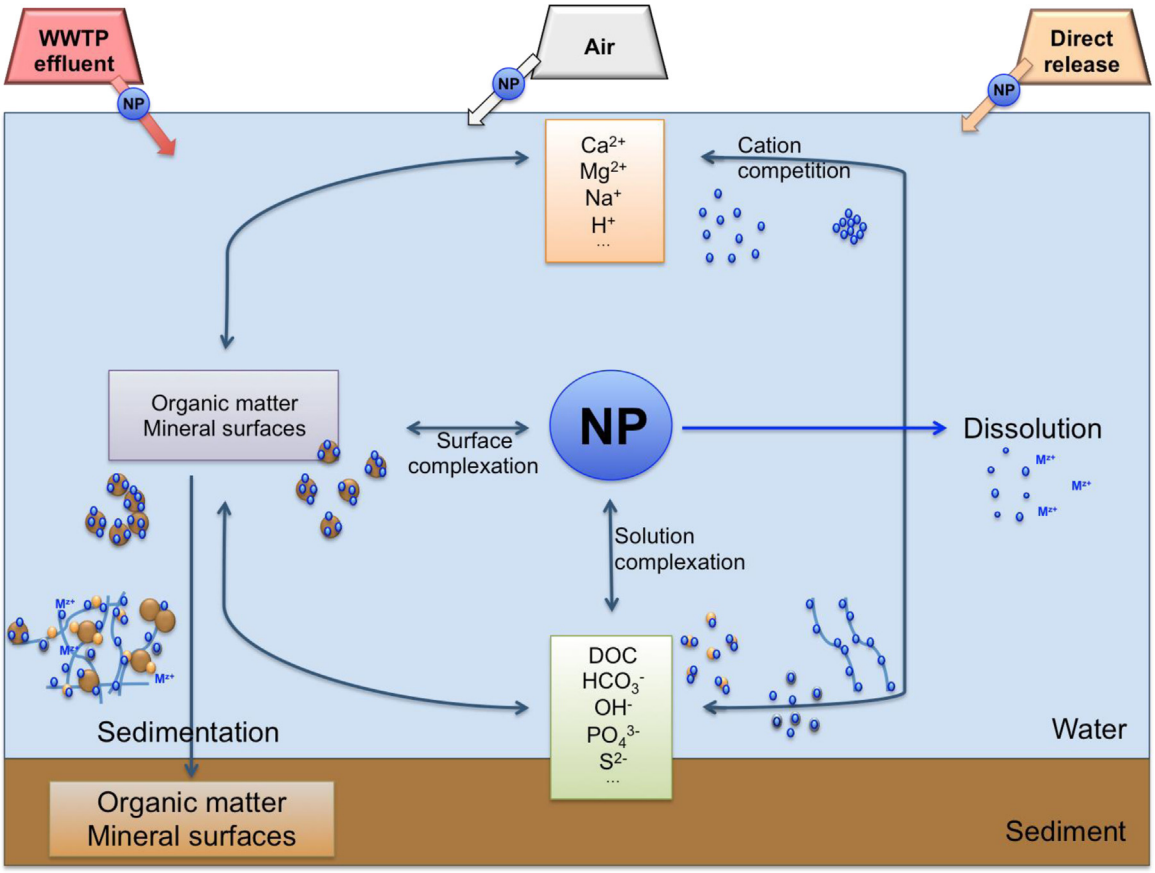

Fig. 1. Representative chemical and physical transformations of NPs when entering in natural aquatic systems: dissolution, phosphatization, sulfidation, homo- and heteroaggregation, and sedimentation. Important constituents with which NPs can interact governing their fate and transport includes hardness cations (e.g., Ca^{2+}, Mg^{2+}), alkalinity, phosphate and sulfide anions, pH, dissolved organic carbon (DOC), organic matter (OM) and mineral surfaces (such as iron and manganese oxides, and clays). Legend: blue circles: engineered NPs; yellow circles: humic substances (HM); brown circles: natural inorganic colloids; blue lines: rigid biopolymers; gray surroundings: representing sulfidation; M^{z+}: free metal ion. Adapted from Domingos et al. (2015). For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

bioavailability to the organisms; when aggregates become too large for direct transport across the cell wall and/or membrane, uptake may be prevented, whereas partial dissolution, which will lead to smaller sizes, would facilitate this cellular transport. Since these transformations are most often not in equilibrium, they require real-time kinetic measurements, limiting the methodology to be used: (i) the storage of whole unfractionated samples for ion analysis may not be possible since the dissolution rate may be fast or not attain the equilibrium during the experimental time, and (ii) the aggregation rate can be fast or the aggregates size distribution may not reach equilibrium within the experimental time window.

Despite the large number of studies focused on nanotoxicology, most of them disregard the particles kinetic physicochemical characterization under the exposure conditions, hindering the establishment of crucial predictive structure-activity relationships that can be used afterwards in the categorization and function for risk assessment studies. In absence of these realistic studies, two less constrain conditions were used to select the literature studies that will be discussed in this critical overview:

i) studies where the NPs characterization was performed in the same media as the bioassays.
ii) quantification of the NPs dissolution for studies using metallic NPs with propensity for dissolution such as nAg and nZnO.

3. Nanotoxicity toward aquatic organisms

3.1. Generation of ROS

Despite the large number of studies on NPs toxicity both in cell line systems and organisms, a complete understanding about the mechanisms behind is still lacking (see reviews (Manke et al., 2013; Fu et al., 2014; Bour et al., 2015; Schultz et al., 2014)). ROS generation, whose overproduction can lead to oxidative stress (OS) in the organism tissues, is unquestionably the most studied nanotoxicity mechanism.

Molecular oxygen is used as an oxidizing agent for the production of adenosine triphosphate (ATP) in the organism cells, being afterwards reduced to water. The non reduced oxygen results in the formation of superoxides $\left(O_2^{-\bullet}\right)$ that can be further converted to hydroxyl radicals ($HO^{\bullet}$), which has the highest reduction potential of all the physiological relevant ROS. When under control, these species are easily scavenged by (i) antioxidant agents such as polyphenols (Fu et al., 2014; Lipinski, 2011) (e.g., elimination of $HO^{\bullet}$), and (ii) enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The SOD enzymes catalyze the dismutation of $O_2^{-\bullet}$ into oxygen or hydrogen peroxide (H_2O_2), which is decomposed by CAT into water and oxygen. Even though H_2O_2 is less reactive than the radical species, is still a strong oxidant that needs further elimination. The GPx, also plays a role in the detoxification of H_2O_2 by using glutathione

(GSH) as a reductant. During the process, GSH is oxidized and converted to glutathione disulfide (GSSG) being latter reduced back to GSH by glutathione *s*-transferase enzymes (GST), thus completing the cycle (Brigelius-Flohe and Maiorino, 2013; Deponte, 2013). The antioxidant-enzymes activity is considered a reflection of the redox state of the cells and is frequently studied as a biomarker of OS.

When ROS is overproduced beyond the organism antioxidant response capacity, it leads to several deleterious effects on the cells components such as lipids, proteins and DNA, possibly resulting in lipid peroxidation, apoptosis and/or cancer initiation processes, respectively. The production of ROS can be enhanced by the presence of NPs, depending mainly on their size, aggregation, solubility and coating. It is commonly accepted that smaller particles can easily penetrate cell membranes, and thus induce cytotoxicity (Sakai et al., 2011; Buzea et al., 2007). However, this correlation between size and toxicity is still controversial. Shi et al. (2013) reported that 5–10 nm nAg had higher toxicity to *Tetrahymena pyriformis* when compared to slightly larger nAg (15–25 nm), while no size-dependent response on *Danio rerio* was obtained when exposed to 20, 50 and 110 nm (Bowman et al., 2012). Most studies show that toxicity increases with decreasing particle size. However, others reported that either the size has no role on toxicity or that smaller NPs are less toxic (Ivask et al., 2014). A consensus about the size effect is still lacking, and, most probably, will be unlikely to be attained since the effects seem to be NP- and even specie-dependent (Buric et al., 2015).

The dissolution of metallic NPs such as nCuO and nAg results in the release of Cu and Ag ions, which are known to catalyze Fenton, Fenton-like and Haber–Weiss reactions, leading to the formation of ROS (Fu et al., 2014; Lipinski, 2011; He et al., 2012; Wang et al., 2013b,c). Moreover, the highly reactive surface of NPs and the presence of manufactured and/or natural coatings can lead to the adsorption/complexation of trace metals present in the environmental compartment also acting as a catalyzer platform to the above-mentioned reactions, and thus increasing the concentration of ROS in the system. Photoactive NPs such as $nTiO_2$ and nZnO, can also induce the formation of ROS. When exposed to visible or UV light, these NPs can be photo excited resulting in the formation of electron–holes, which are powerful oxidants that can react with surface bounded molecules forming radicals (Clemente et al., 2014). All these processes are schematized in Fig. 2.

3.2. Omics endpoints

The omics tools, such as toxicogenomic, metallomic and proteomic, are very useful on the establishment of toxic endpoints. A toxicogenomics approach allows the identification of gene and protein activities in the organisms cells induced when in the presence of a certain xenobiotic. A central assumption is that chemicals generating toxicity by the same mechanism will produce similar gene expression responses under a given set of conditions, bringing new insights about their mode of action that can be linked to their specific physicochemical properties. A metallomic and proteomic approach will allow a complete analysis on the metal and metalloid species composition within a cell or tissue and the establishment of metalloproteins profiles leading to the identification of new biomarkers (e.g., proteins expressed by the NP itself (Shepard et al., 2000)). This will allow a better understanding and profiling of NPs toxic mechanisms discriminating them from their bulk components.

Despite the large number of studies about nanotoxicology, only a few number have reported the use of omics tools to evaluate the NPs toxic effects at the molecular level on freshwater organisms (Rainville et al., 2014; Kuznetsova et al., 2014). Indeed, the "omics" approach generates a huge amount of data whose interpretation is not always straightforward, being, most probably, the main reason moving nanotoxicologists away from using these tools. This large amount of data together with the fact that usually an appropriate physicochemical characterization prior to the biological assays is not performed, results in an escalating number of unknown variables impeding a comprehensible understanding of the biochemical responses.

4. NPs toxicity on freshwater organisms

4.1. Silver NPs (nAg)

nAg are known for their antifungal and antimicrobial properties, being extensively used in several products such as clothing, cosmetics, medical devices, paints, humidifiers and refrigerators (Reidy et al., 2013; Fabrega et al., 2011), leading to predicted concentrations of 0.088–2.16 ng L^{-1} in European and North American surface waters (Gottschalk et al., 2009).

Despite nAg is one of the most studied NPs, the toxicity mechanisms are still not fully clear; some assign the nAg toxicity to the release of Ag^+ ions to the media, while others assume that intact nAg particles are responsible for the induction of toxic responses in the organisms (Schultz et al., 2014). It is well known that Ag^+ ions has a great propensity to bioaccumulate in the tissues leading to ROS generation, genotoxicity and inhibition of Na^+/K^+-ATPase activity by blocking the Na^+ uptake by the cells (Luoma, 2008; Morgan et al., 1997). Similar toxic effects were also observed in juvenile *Oncorhynchus mykiss* fishes exposed to citrate coated nAg, with claimed low propensity for dissolution (Schultz et al., 2012). However, it was not possible to confidently establish if these inhibitions were caused by the nAg per se or by dissolved Ag^+. In fact, it is very difficult to distinguish between the toxic effects induced by particulate or ionic Ag, and, thus, is crucial not only to evaluate the size distribution but also to quantify the nAg dissolution in the bioassay (Table 1).

It was possible to identify two distinct routes that could induce biochemical responses in freshwater organisms (Table 1) (1) presence of the NPs per se, and (2) presence of both nAg and dissolved Ag^+. For the first route the identification of responses that are uniquely due to the presence of nAg and do not occur in matched Ag^+ exposures is crucial. This is possible by (i) using nAg with low dissolution rates, so, that the leached Ag^+ in the media is insufficient to induce toxicity to the organisms, or (ii) identifying endpoints specific to nAg, such as internalization of NPs, cytotoxicity and genotoxicity. The work performed by Kumar et al. (2014) is an example on the application of these strategies. They reported a significant increase of ROS and SOD and morphological alterations on freshwater bacteria exposed to nAg coated with PVP. Since the NPs dissolution was very low (leached $Ag^+ < 1$ μg L^{-1}), it was concluded that the results were related to the silver particulate form. Morphological changes and alteration in genes profiles, related to the presence of nAg itself, were also observed in *Cyprinus carpio* (Lee et al., 2012), zebrafish (Griffitt et al., 2009; Choi et al., 2010), *Daphnia magna* (Poynton et al., 2012), medaka fish (Pham et al., 2012) and rainbow trout (Gagne et al., 2012). The exposure of 7 day old *D. magna* to citrate-coated nAg (size 10 nm, 30 μg L^{-1}) and $AgNO_3$ (2.5 μg L^{-1}) during 24 h showed that both Ag forms act through different pathways; both forms increased proteins thiol content, but only particles increased proteins carbonyl levels (Gundel et al., 2007).

For the second route, both forms, particulate and ionic Ag, can induce OS and genotoxicity being the distinction between these effects a truly challenge. For example, in *Chlorella vulgaris* a positive correlation between ROS production and LPO on the tissues after 24 h of exposure to uncoated nAg (1 and 10 mg L^{-1}) (Oukarroum et al., 2012) was found, but with no possibility to establish a positive

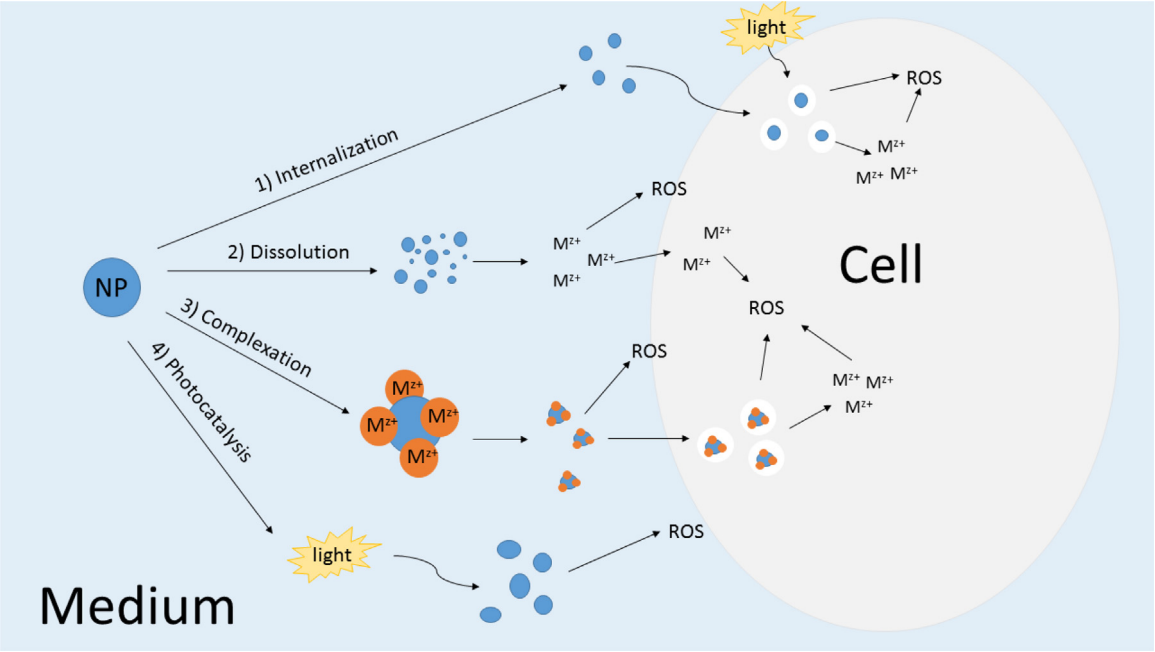

Fig. 2. Potential routes for the generation of ROS due to the presence of NPs. (1) Internalization of NPs–ROS generation could occur due to the NPs dissolution inside the cells and/or due to the NPs photocatalytic activity. (2) Dissolution of the NPs leads to an increase concentration of metal ions in the media; some of these metals can also be uptake by the organisms. (3) NPs and/or their surrounding coatings can adsorb/complex other metals present in the media, being taken up by the cells. (4) Photocatalytic activity of the NPs in the presence of UV and/or natural light.

correlation with solely the particulate or ionic Ag. Similar difficulties were also observed in the snail *Lymnaea luteola* (Ali et al., 2014), where the exposure to nAg resulted in DNA damage and induction of OS but without the possibility to identify which Ag forms has the dominant role on the observed effects. In these situations, a complete physicochemical characterization of the NP in the exposure media along with the use of controls containing ionic Ag are crucial to understand which Ag form (or both) is responsible for the observed biochemical responses.

Although in the majority of the studies is not possible to establish if the nAg potential toxicity is due to its particulate or ionic form, it is clear that the presence of nAg in freshwater systems presents a high risk to aquatic life.

4.2. Titanium dioxide NPs ($nTiO_2$)

$nTiO_2$ is one of the most produced NPs in the world, with an expected production of 201,000 tons during 2015 (Epa, 2011; Markets, 2015). Some studies have reported evidences that this NP have a low toxicity toward aquatic organisms even at concentrations higher than the ones expected to occur in the freshwater systems (3 ng L^{-1} to only 1.6 μg L^{-1}) (Gottschalk et al., 2013). Federici et al. (2007) have showed that even at 0.1–1 mg L^{-1} of $nTiO_2$ the rainbow trout, defense system can naturally scavenge ROS species avoiding OS. For this reason, most of the nanotoxicology studies (if not all) uses much higher concentrations with the objective of obtain more straightforward acute toxicity responses. Evidently, as used NPs concentrations are largely higher than the above predicted values, the environmental impact of these studies decreases significantly. Moreover, higher $nTiO_2$ concentrations impacts on their own undergone transformations; the presence of a higher number of particles can lead to homoaggregation (see Section 2), most probably, resulting in a misinterpretation of the obtained results. Dalai et al. (2013) showed that for $nTiO_2$ concentrations larger than 16 mg L^{-1}, ROS levels in the *Ceriodaphnia dubia* decreased significantly due to the agglomeration of the NPs becoming less bioavailable to the organisms. The exposure of *O. mykiss* (rainbow trout) to fairly low concentrations of $nTiO_2$ (0.1–1 mg L^{-1}) resulted in biochemical disturbances, respiratory distress and several organ pathologies (Federici et al., 2007; Boyle et al., 2013). However, the same did not occur, when other organisms were exposed to higher concentrations (Dalai et al., 2013; Hao et al., 2009). Table 2 resumes the biochemical responses of freshwater organisms, mostly daphnids and fishes exposed to $nTiO_2$. In most of these studies, the $nTiO_2$ characterization was solely based on the evaluation of the NPs size distribution since aggregation is the most important transformation for this particle.

Three main routes leading to $nTiO_2$ toxicity were identified: (i) physical stress, associated with the NPs size and surface properties (cytotoxicity), (ii) photocatalytic activity (phototoxicity), and (iii) NPs capacity do adsorb xenobiotics in the media.

The internalization of $nTiO_2$ by an organism may result in its accumulation in different organs leading to physical stress and tissues damage. For instance, accumulation of $nTiO_2$ in daphnid's guts (Dalai et al., 2013; Tan and Wang, 2014; Zhu et al., 2010), bivalve's digestive gland (Vale et al., 2014) and fish's gills (Federici et al., 2007; Boyle et al., 2013; Hao et al., 2009) constricts their alimentary canal affecting their breathing capacity. The possible mechanisms involved in the NPs internalization in the organisms were already described (Reidy et al., 2013; Chen and Bothun, 2014; Shang et al., 2014), and will not be further discussed here.

Due to their photocatalytic activity, $nTiO_2$ can generate radical species when exposed to natural and/or UV light radiation. A linear relationship between illumination and the hydroxyl radical generation in the media was established in presence of $nTiO_2$, with the extracellular radical species generated inducing oxidative damage on the gill tissue cells of *D. rerio* (Xiong et al., 2011). Similar effects were also observed in other freshwater organisms such as zebrafish (Bar-Ilan et al., 2013), rainbow trout (Boyle et al., 2013) and daphnids (Dalai et al., 2013). Moreover, $nTiO_2$ can lead to toxicity assumed to be promoted by OS under dark conditions in laboratory and in microcosm study (Jomini et al., 2012).

The $nTiO_2$ can adsorb or interact with different elements, (e.g., Cd, Zn, Pb, Cu, Ni and As (Vale et al., 2014; Engates and Shipley,

Table 1
Impact of nAg in freshwater organisms, including physicochemical characterization of the NPs in the media and biochemical responses. The biomarkers of response expressed as ↑ and ↓, indicates an increase or decrease on the biomarkers activity or concentration, respectively, when compared with the control groups (organism not exposed to nAg). The biomarkers here listed are: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione-s-transferase (GST), total glutathione peroxidase (GPx), metallothioneins (MTs), lipid peroxidation (LPO) and reactive oxygen species (ROS).

Supplier information (supplier; coating)	nAg characterization (Size (nm); concentration ($mg\,L^{-1}$); medium)	Bioassay			
		Organism	Medium	Biomarkers of response (nAg concentration ($mg\,L^{-1}$))	Ref.
Sigma–Aldrich uncoated	260 ± 26 (DLS)[a]; 0.001–0.08; TW[c] 32 (TEM)[b]; 0.001–0.08; TW[c]	*Lymnaea luteola* L.	TW[c]	After 24 h exposure: ↓GSH (0.012; 0.024; 0.036); ↑CAT (0.012; 0.024; 0.036); ↓GPx (0.024; 0.036); ↓GST (0.036); ↑LPO (0.012; 0.024; 0.036); DNA damage After 96 h exposure: ↓GSH (0.012; 0.024; 0.036); ↑CAT (0.012; 0.024; 0.036); ↓GPx (0.024; 0.036); ↓GST (0.024; 0.036); ↑LPO (0.004;0.012; 0.024; 0.036); DNA damage	Ali et al. (2014)
MTI Corporation uncoated	307 (DLS)[a]; 1; BG-11[d] 50 (TEM)[b] BG-11[d]	*Chlorella vulgaris*	BG-11[d]	↑ROS (1 and 10); ↑LPO (1 and 10)	Oukarroum et al. (2012)
ABC Nanotech citrate-capped	70 (DLS)[a]; 5; FW [e] 90 (DLS)[a]; 10; FW [e] 12 (TEM)[b]; 5 and 10; Freshwater [e]	*Cyprinus carpio*	FW[e]	Brain: ↓GST (0.1 and 0.2) Liver: ↑GST (0.05; 0.1 and 0.2); ↓CAT (0.025; 0.05 and 0.1)	Lee et al. (2012)
Sigma– Aldrich PVP coated	443 ± 15 (DLS)[a]; 1; FSLW[f] 20-100 (TEM)[b]: 1; FSLW[f]	*B. thuringiensis* and *B. aquimaris*	FSLW[f]	↑ROS (1) ↑SOD (1)	Kumar et al. (2014)
Nanopoly uncoated	5–20 (TEM)[b]	*D. rerio*	BW[g]	↑LPO (60,120) ↑GSH (120) ↓CAT (60,120) ↓GPx (120)	Choi et al. (2010)

[a] Dynamic light scattering.
[b] Transmission electron microscopy.
[c] Tap water.
[d] Culture medium for Cyanobacteria (Rippka et al., 1979).
[e] Freshwater.
[f] Filtered and sterile lake water.
[g] Bottled water.

2011; Gao et al., 2004; Sun et al., 2006)), changing their speciation in the media and affecting their bioavailability and toxicity to organisms. For instances, it was shown a correlation between the decrease on Cd toxicity to the green algae *Pseudokirchneriella subcapita* and the presence of $nTiO_2$ not internalized by the algae. But on the other hand, if $nTiO_2$ is already present in the organism tissues, an increased bioaccumulation of xenobiotics due to adsorption onto the internalized NPs can occur (Tan and Wang, 2014). Dispersed $nTiO_2$ can also act as a carrier of xenobiotics in the media increasing the metal uptake rate (Zhang et al., 2007b).

Independently of the route taken, $nTiO_2$ toxicity is mostly associated with ROS generation, and the most common biochemical endpoints are related with the anti-oxidant enzymes activity (see Table 2). In *D. magna,* an increase of CAT, GST and GPx activity was observed when animals were exposed to 5–10 $mg\,L^{-1}$ of $nTiO_2$ (Kim et al., 2010), whereas in *C. dubia* an increase in SOD activity was also observed after exposure to 1–64 $mg\,L^{-1}$ $nTiO_2$ (Dalai et al., 2013). Similar responses were also found in fishes, *D. rerio* (Clemente et al., 2014; Xiong et al., 2011), *C. carpio* (Hao et al., 2009) and *O. mykiss* (Federici et al., 2007).

4.3. Zinc oxide NPs (nZnO)

As for $nTiO_2$ also, nZnO are among the most used NPs with an estimated production of 30,000 metric tons per year (2015), originating predicted environmental concentrations in surface waters ranging from 0.008 to 0.055 $\mu g\,L^{-1}$ in Europe and 0.001 to 0.003 $\mu g\,L^{-1}$ in US (Gottschalk et al., 2013, 2009). However, and as for the other NPs, the concentrations used in ecotoxicology studies are in general far higher than the ones predicted (Table 3).

Besides being an essential microelement, when at higher concentrations Zn^{2+} is known to be toxic to aquatic organisms (Brun et al., 2014; Mortimer et al., 2010a). Being dissolution one of the main transformations of this particle is necessary to consider that toxicity effects may be provoked by the NP per se and/or by the ionic fraction (Bondarenko et al., 2013; Franklin et al., 2007). Whereas dissolution of nZnO is hindered by the increase of the particle size or agglomerates size (Brun et al., 2014), the presence of proteins can enhance it due to the proteins binding ability toward Zn (Reed et al., 2012). The presence of manufactured stabilizers can also greatly affect the NPs dissolution, with largest dissolved Zn usually observed for uncoated nZnO followed by polymer-stabilized par-

Table 2
Impact of $nTiO_2$ in freshwater organisms, including physicochemical characterization of the NPs in the media and biochemical responses. The biomarkers of response expressed as ↑ and ↓, indicates an increase or decrease on the biomarkers activity or concentration, respectively, when compared with the control groups (organism not exposed to $nTiO_2$). The biomarkers here listed are: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione-s-transferase (GST), total glutathione peroxidase (GPx), metallothioneins (MTs), lipid peroxidation (LPO) and reactive oxygen species (ROS).

Supplier information (supplier; chrystal phase)	$nTiO_2$ characterization (Size (nm); concentration ($mg\,L^{-1}$); medium)	Bioassay			
		Organism	Medium	Biomarkers of response ($nTiO_2$ concentration ($mg\,L^{-1}$))	Ref.
Sigma–Aldrich 30% rutile + 70% anatase	400–800 (DLS)[a]; 5; MHW[b]	*D. magna*	MHW[b]	↑CAT (10); ↑GST (5 and 10); ↑GPx (5 and 10)	Kim et al. (2010)
Sigma–Aldrich 100% anatase	400–700 (DLS)[a]; 100; EEM[c]	*D. rerio*	EEM[c]	Visible light: ↓CAT (1 and 10); ↑GST (10) UV + visible light: ↓GST (10)	Clemente et al. (2014)
Sigma–Aldrich 100% anatase	20.9 ± 2.86 (DLS)[a]; 0.1; SM7[d] 218 ± 47.3 (DLS)[a]; 1; SM7[d] 21 (TEM)[d];; 0.1 and 1; SM7[d]	*D. magna*	SM7[d]	No significant effects on ROS and MTs	Tan and Wang (2014)
Sigma–Aldrich 100% anatase	248–293(1); FSLW[e] 517–925 (16); FSLW[e] 697–1090 (64); FSLW[e]	*C. dubia*	FSLW[e]	Photoperiod (16 h light: 8 h dark): ↑SOD (1–64); ↑ROS (1–32); ↓ ROS (>32) Dark period: ↑SOD(1–64); ↑ROS (1–64)	Dalai et al. (2013)
Degussa Evonik 80% rutile + 20% anatase	>1000 (DLS)[a]; 100; EEM[c]	*D. rerio*	EEM[c]	UV + visible light: ↓CAT (1 and 10); ↓GST (1)	Clemente et al. (2014)
Degussa Evonik 75% rutile + 25% anatase	24 (TEM)[f]; 0.1;0.5 TW[g]	*O. mykiss*	TW[g]	Liver tissues: ↓GSH (0.5 and 1) Gill tissues: ↑ GSH (1); ↑LPO (0.1 and 5) Brain tissues: ↑LPO (0.1; 0.5 and 1) Intestine tissues: ↑LPO (0.1; 0.5 and 1)	Federici et al. (2007)
Nanjing University 100% anatase	249–488 (DLS)[a]; 1; DW[h] 270–535 (DLS)[a]; 10; DW[h] 251–630 (DLS)[a]; 50; DW[h] 402–633 (DLS)[a];100; DW[h] 245–617 (DLS)[a]; 300; DW[h] 20–70 (TEM)[f]; DW[h]	*D. rerio*	DW[h]	Liver tissues: ↓CAT (50), ↓SOD (50), and ↓GSH (50) Gut tissues: ↑SOD (50); ↑GSH (50); ↑LPO (50) Gill tissues: ↑LPO (50)	Xiong et al. (2011)

[a] Dynamic light scattering.
[b] Moderately hard water.
[c] Embryo exposure medium.
[d] Elendt simplified M7 medium.
[e] Filtered and sterile lake water.
[f] Transmission electron microscopy.
[g] Tap water.
[h] Distillated water.

ticles (Merdzan et al., 2014). These NPs can also dissolve after its internalization in the extra- or intracellular compartments (Fig. 2), dramatically changing the organism's metallome profile.

The exposure of the algae *P. subcapitata* to nZnO, $ZnSO_4$, $ZnCl_2$ and bulk ZnO resulted in similar toxic effects indicating that ionic Zn was the responsible for the observed toxicity (Franklin et al., 2007; Aruoja et al., 2009a; Neale et al., 2015). Dose–dependent adverse effects of nZnO on *D. rerio* embryos and eleuthero-embryos were observed (Brun et al., 2014), resulting in hatching and inflammation reactions (Yu et al., 2011; Xia et al., 2011). Zn may act as a competitor with Ca influx in skin chloride cells by blocking its transport through the pore canals and affecting a large surface of the skin. The observed toxic effects were related to the ionic Zn, since the NPs size distribution was too large to pass through the chorion pores. In contrast, zebrafish embryos showed a greater acute toxicity to nZnO than when in presence of ionic Zn (Zhu et al., 2008).

nZnO can establish hydrogen bonds and ligand exchanges with the bacterial cell wall, modifying the protein structure and consequently changing its function (Jiang et al., 2010). These particles can also bind to the cytoplasmic bacterial membrane possibly disrupting its integrity, and interrupt the fundamental role of electron transport phosphorylation and energy transduction process (Lyon et al., 2007). nZnO can generate ROS due to its photocatalytical properties (Suresh et al., 2015), but can also suffer photocorrosion

Table 3
Impact of nZnO in freshwater organisms including physicochemical characterization of the NPs in the media and biochemical responses. The biomarkers of response expressed as ↑ and ↓, indicates an increase or decrease on the biomarkers activity or concentration, respectively, when compared with the control groups (organism not exposed to nZnO). The biomarkers here listed are: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione-s-transferase (GST), total glutathione peroxidase (GPx), lipid peroxidation (LPO), nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor α (TNFα), myxovirus-resistance protein A (MxA), interleukin-1 beta (IL-1β), mitochondrial uncoupling protein 2 (Ucp-2), heat shock proteins (HSP) and macrophage scavenger receptor (MSR).

Supplier information (supplier; chrystal phase)	nZnO characterization (Size (nm); concentration ($mg\,L^{-1}$); medium)	Bioassay			
		Organism	Medium	Biomarkers of response ($nTiO_2$ concentration ($mg\,L^{-1}$))	Ref.
Genes'Ink	218–316 (NTA)[a]; 5; HM [b] 196–211 (NTA)[a]; 0.2; HMA[c] 214–236 (NTA)[a]; 1; HMA [c] 223–242 (NTA)[a]; 5; HMA[c]	*D. rerio* (embryos)	HMA[c]	↑ CAT (0.2, 1 and 5) 48 hpf; ↓ CAT (5) 96 hpf ↑ Cu/Zn-SOD (5) 48 hpf; ↓ Cu/Zn-SOD (5) at 96 hpf ↑ mt2 gene (0.2, 1 and 5) 48 and 96 hpf ↓ TNFα (5) 96 hpf ↑ c-jun protein (0.2, 1 and 5) 96 hpf ↓ MxA (1 and 5) at 48 hpf; ↓ MxA (5) 96 hpf ↓ IL-1β (5) 96 hpf	Brun et al. (2014)
Genes'Ink	218–316 (NTA)[a]; 5; HMWA[b] 196–211 (NTA)[a]; 0.2; HMA[c] 214–236 (NTA)[a]; 1; HMA[c] 223–242 (NTA)[a]; 5; HMA[c]	*D. rerio* (eleuthero-embryos)	HMA [c]	↑ CAT (0.2 and 1) 120 hpf ↑ mt2 gene (5) 120 and 168 hpf ↑ TNFα (1 and 5) 120 hpf ↓ Cu/Zn-SOD (5) 120 hpf ↓ c-jun protein (1 and 5) 120 and 168 hpf, ↓ MxA (5) 120 and 168 hpf, ↓ IL-1β (1 and 5) 120 and 168 hpf	Brun et al. (2014)
Nanjing High Technology	2196–3144 (DLS)[d]; 10; ZCM[e]	*D. rerio* (embryos)	ZCM[e]	↑ ROS (1 and 10) at 96 hpf ↓ GSTp2 (10) at 96 hpf ↓ Nqo1 gene (10) at 24 and 96 hpf ↑ GSTp2 and Nqo1 gene (sediment + nZnO 10) at 96 hpf	Zhu et al. (2009)
Sigma–Aldrich	50–100 (DLS)[d]; 50; ZCM[e]	*D. rerio* (embryos) at 144 hpf	ZCM[e]	↑ SOD (1–100, dose–dependent from 1 to 50) ↑ MDA (20–100); ↑ GPx (20–100); ↑ DNA damages (100) ↑ ROS (1 and 10; ↑ Ucp-2 (1–100); ↓ CAT (100); ↓ GSTp2 (50–100); ↓ Nqo1 gene (100); ↓ Bcl-2 genes (50–100)	Zhao et al. (2013)
BASF UK Z-COTE©	150 ± 60 (TEM)[f]; 100; NH[g]	*E. coli*	NH[g]	↑ SoxS (100)	McQuillan and Shaw (2014)
Nanostructured & Amorphous Material, Inc.	24–72 (FCS)[h]; 1; EP[i] 10–13 (AUC)[j]; 1; EP[i]	*C. reinhardtii*	EP[i]	Moderate Visible light: ↑ GSTs1 (1); ↑ HSP22C (1); ↑ HSP70A (1); ↑ MSR1(1); ↑ MSR2(1); and ↑ HSP90(1)	Simon et al. (2013)
nCuO; Nanostructured & Amorphous Materials, Inc.	~130 (DLS)[a]; 10; MES[b] ~140 (DLS)[a]:10; MOPS[c] ~140 (DLS)[a];10; TAP[d] ~140 (DLS)[a];10; OECD[e] ~140 (DLS)[a]; 10; LW[f]	*Chlamydomonas reinhardtii*	MES[b]; MOPS[c]; TAP[d]; OECD[e]; LW[f]	↑ROS showing OECD[e] > MOPS[c] > MES[b] > TAP[d] > LW[f] after 24 h	von Moos et al. (2015)
MTI Corporation	148(DLS)[a]; 40; HSM[g]	*C. reinhardtii*	HSM[g]	↑ROS (0.004 g L^{-1})	Perreault et al. (2012)
Nanostructured & Amorphous Material, Inc.	27.2 ± 6.7 (TEM)[f]; 22; MHRW[k]	*D. magna*	MHRW[k]	↓ Ferritin 3 (2.2 and 9.0); ↓ C1q proteins (2.2 and 9.0)	Poynton et al. (2011)

Table 3 (*Continued*)

Supplier information (supplier; chrystal phase)	nZnO characterization (Size (nm); concentration ($mg\,L^{-1}$); medium)	Bioassay			
		Organism	Medium	Biomarkers of response ($nTiO_2$ concentration ($mg\,L^{-1}$))	Ref.
Sigma–Aldrich	264.8 (DLS)[d]; 1000; DW[l]	*L. luteola* L.	DW[l]	Digestive gland cells ↑ DNA Damage (10–32) 24 h and 96 h ↑ LPO (21–32) 24 h; ↑ LPO (10–32) 96 h ↑ CAT (21–32) 24 h and 96 h ↓ GPx (21–32) 24 h; ↓ GPx (10–32) 96 h ↓ GST (21–32) 24 h; ↓ GST (10-32) 96 h ↓ GSH (21–32) 24 h and 96 h	Ali et al. (2012)
Sigma–Aldrich	71.11 (DLS)[d]; 1000; DW[l]	*B. alexandrina*	DW[l]	Hemolymph: ↓ CAT (7–35); ↓ SOD (7) and ↑ SOD (35); ↓ GSH (7–35); ↓ GST (7–35); ↑ NO (7–35) ↑ LPO (7–35) Tissues: ↓ CAT (7) and ↑ CAT (35); ↓ SOD (7) and ↑ SOD (35); ↓ GSH (7–35); ↓ GST (7–35); ↑ NO (7–35) ↑ LPO (7–35)	Fahmy et al. (2014)

[a] Nanoparticle tracking analysis.
[b] Holtfreter's medium.
[c] Holtfreter's medium with alginate.
[d] Dynamic light scattering.
[e] Zebrafish culture medium.
[f] Transmission electron microscopy.
[g] Neidhardt's medium.
[h] Fluorescence correlation spectroscopy.
[i] Experimental media.
[j] Analytical ultra centrifugation (Saison et al., 2010).
[k] Moderately hard reconstituted water.
[l] Destilated water.

when exposed to UV light, decreasing their photocatalytic activity in aqueous suspensions (Hariharan, 2006). OS induced by both nZnO and/or released Zn^{2+}, has already been observed in several freshwater organisms, such as bacteria (Lyon et al., 2007; Cabiscol et al., 2000; Adams et al., 2006; Zhang et al., 2014; Gunawan et al., 2013), algae (Simon et al., 2013), crustacean (Mwaanga et al., 2014), mussel (Gagné et al., 2013), snail (Fahmy et al., 2014), frog (Bacchetta et al., 2012, 2013; Nations et al., 2011) and fish (Brun et al., 2014; Xia et al., 2011; Hao and Chen, 2012; Zhu et al., 2009; Bai et al., 2010; Zhao et al., 2013; Nel et al., 2006). In bacteria, the OS effect of nZnO may occur under dark conditions as well as under natural or artificial light and affect both gram-positive and negative bacteria (Barnes et al., 2013). After exposure to UV light radiation, a loss of viability in *Escherichia coli* and ROS generation results in the expression of genes encoding SoxS and CAT, which are part of the OS response mechanism (Gunawan et al., 2013; Kumar et al., 2011). In the algae *Chlamydomonas reinhardtii*, the presence of nZnO led to elevated levels of transcripts genes, *GSTS1*, *HSP22C*, and *HSP70A*, and the transcripts encoding sub units *MSR1*, *MSR2*, and *HSP90* which are involved in the organism defense response against ROS (Simon et al., 2013). In bivalves, the presence of nZnO led to an increase of LPO in the digestive gland (Gagné et al., 2013). Similar findings were also observed in snails (Fahmy et al., 2014), with a decrease in GSH content, a significant inhibition of SOD and CAT activity and a decrease in total protein and albumin contents. A significant increase of LPO levels and a decrease of GSH activity in the gills, liver and brain of carps were also reported after a 14 days exposure to 50 mg nZnO L^{-1} (Hao and Chen, 2012). A concentration-time exposure effect on SOD, CAT, GPx, and Ucp-2 expression with an increase in MDA content was shown for zebrafish embryos (Brun et al., 2014; Zhao et al., 2013).

As found for $nTiO_2$, nZnO can also adsorb several elements such as As, Al, Mo, Hg, Pb, Cu, Ni and Cd (Gagné et al., 2013; Ghiloufi, 2013; Hua et al., 2012; Mahdavi et al., 2012; Sheela et al., 2012), changing their speciation in the media and thereby their bioavailability. When internalized, these NP-metal complexes may undergo dissociation followed by NPs dissolution due to the acidic conditions found internally resulting in the release of the adsorbed xenobiotics, and drastically increasing their concentration in organism tissues.

Overall, these studies highlight the fact that, despite the vast literature, more investigation is needed to fully elucidates the mechanisms of nZnO toxicity from the cellular level to the organisms or population level.

4.4. Copper oxide NPs (nCuO)

nCuO is a widely used metal oxide presenting biological activity (Moschini et al., 2013; Blinova et al., 2010; Chen et al., 2008;

Table 4
Impact of nCuO in freshwater organisms including physicochemical characterization of the NPs in the media and biochemical responses. The biomarkers of response expressed as ↑ and ↓, indicates an increase or decrease on the biomarkers activity or concentration, respectively, when compared with the control groups (organism not exposed to nCuO). Lipoprotein (LP) and reactive oxygen species (ROS).

Supplier information (supplier)	nCuO characterization (Size (nm); concentration ($mg\,L^{-1}$); medium)	Bioassay			
		Organism	Medium	Biomarkers of response (nCuO concentration ($mg\,L^{-1}$))	Ref.
Nanostructured & Amorphous Materials, Inc.	~130 (DLS)[a]; 10; MES[b] ~140 (DLS)[a]:10; MOPS[c] ~140 (DLS)[a];10; TAP[d] ~140 (DLS)[a];10; OECD[e] ~140 (DLS)[a]; 10; LW[f]	*Chlamydomonas reinhardtii*	MES[b]; MOPS[c]; TAP[d]; OECD[e]; LW[f]	↑ROS showing OECD[e] > MOPS[c] >MES[b] > TAP[d] > LW[f] after 24 h	von Moos et al. (2015)
MTI Corporation	148(DLS)[a]; 40; HSM[g]	*C. reinhardtii*	HSM[g]	↑ROS (0.004 $g\,L^{-1}$)	Perreault et al. (2012)
Sigma–Aldrich	209 ± 10 (DLS)[a]; 100; MQW[h]	*T. thermophila*	OMM[i]	↑ROS (80); ↑LP (60)	Mortimer et al. (2011)
	1230 ± 200 (DLS)[a]; 100; OMM[i]				
	25.5 ± 0.8 $m^2\,g^{-1}$ (BET)[j]				
Sigma–Aldrich	30 (SEM)[k]	*T. thermophila* (strain BIII)	OMM[i]	Cytotoxicity: fluorescence (4 h: 127 (124–144); 24 h: 97.9 (80.4–138) mg Cu L^{-1}); ATP (4 h: 129 (111–149); 24 h: 101 (91.1–190a mg Cu L^{-1})	Mortimer et al. (2010b)
MTI Corporation	523-800 (DLS)[a]; NR[m], SFW[n]	*Lemna gibba*	SFW [150]	↑ROS (0.7 $g\,L^{-1}$ of total Cu) > esterase activity	Perreault et al. (2014)
	−39.7 ± 3.8 mV (EPM)[o]; NR[m]; SFW[n]				

[a] Dynamic light scattering.
[b] 2-(*N*-morpholino) ethanesulfonic acid.
[c] 3-(*N*-morpholino) propanesulfonic acid.
[d] TAP x4 algae growth media.
[e] OECD algae growth media.
[f] Lake water (filtered and sterile).
[g] High salt medium (Saison et al., 2010).
[h] MilliQ water.
[i] Osterhout's mineral medium.
[j] Brunauer–Emmet–Teller analysis.
[k] Scanning electron microscopy.
[m] Not reported.
[n] Synthetic freshwater (Frankart et al., 2002).
[o] Electrophoretic mobility.

Midander et al., 2009), and producing DNA damages and cell death compared to micro-sized particles, and $nTiO_2$ and nZnO (Midander et al., 2009; Ahamed et al., 2010; Nel et al., 2009). However, its environmental hazard has been poorly investigated (Kahru and Dubourguier, 2010) as can be observed in Table 4.

The presence of nCuO (0.1 mg Cu L^{-1}) induced formation of superoxide anions, hydrogen peroxide and single-stranded DNA in different recombinant luminescent *E. coli* (Bondarenko et al., 2012). However, the dissolution of these particles was the key factor triggering ROS and DNA damage. In other study is was also observed that the presence of nCuO and released Cu^{2+} induced an approximately 5-fold increase in ROS in *E. coli* compared to the bacteria-only control (Gunawan et al., 2011). The levels of non-viable cells exposed to nCuO and released Cu^{2+} were very similar suggesting that adverse effects were originated by the ionic form as found by (Ivask et al., 2010). Also Hu et al. (2009) showed that particles significantly inhibited viable count of bacteria (36.8 – 81.9%) when using 25–200 mg nCuO L^{-1}.

A low mutagenic potential to *Salmonella typhimurium* TA 97a and TA100 (marginal effects between 100 and 1600 μg/plate) was displayed by nCuO (Pan et al., 2010). The OS provoked by nCuO on diverse *Saccharomyces cerevisiae* strains was investigated and com pared with $CuSO_4$ and bulk CuO (Kasemets et al., 2013). The cup2Δ (Cu stress response-deficient strain) was the most sensitive strain (approximately 16-fold than the wild type), suggesting that nCuO effect proceeds also *via* dissolved Cu-ions. EC50s of nCuO (16–19 mg Cu L^{-1}) and $CuSO_4$ (10–12 mg Cu L^{-1}) differed from the bulk CuO (918–1082 mg Cu L^{-1}). Effects on yeast growth were also reported (Kasemets et al., 2009a).

The paradigm of nCuO OS (10 mg L^{-1}) was also investigated on *C. reinhardtii* (von Moos et al., 2015), with results (24 h exposure) showing an immediate cell size increase, OS, and chlorophyll bleaching, while membrane permeabilization was observed after 5 h. Agglomerated nCuO was toxic and the exposure media was decisive in whether or not particles or ionic Cu act as the main toxicity mediators. *C. reinhardtii* was also used to show the effect of the presence of a manufactured coating on nCuO (polystyrene-co-butyl acrylate) comparing the outcomes with bare nCuO (Perreault et al., 2012). Higher toxicity was obtained in presence of coated nCuO mainly due to its internalization in the cytosolic membrane structures. ROS formation was observed at 4 mg L^{-1} of coated nCuO reaching (392 ± 12)% at 40 mg L^{-1}, whereas 4 mg L^{-1} of bare nCuO formed ROS that increased only up to (160 ± 15)% compared to the control (Perreault et al., 2012). Coated nCuO was also 10-fold more toxic for *Lemna gibba* than bare nCuO (Perreault et al., 2014); the 48 h exposure of 0.4 $g\,L^{-1}$ of coated-nCuO led to a 50% growth inhi-

bition, while 4.5 g L^{-1} of bare nCuO were required to reproduce the same effect. But particles dissolution played the major role in the toxicity.

Tetrahymena thermophila was used to assess nCuO, bulk CuO and Cu salt effects on fatty acid profiling, ROS and LPO (Mortimer et al., 2011). All Cu forms induced ROS, but a larger induction was obtained in presence of nCuO, which could not be explained solely by the released Cu. Protozoa exposure (24 h) to 80 mg L^{-1} of nCuO significantly decreased the proportion of the two major unsaturated fatty acids, increasing the relative amount of two saturated fatty acids. Analogous effects were not observed with other Cu forms. Several studies confirmed that ionic Cu contributed only partially to nCuO toxicity (Shi et al., 2011; Kasemets et al., 2009b; Aruoja et al., 2009b; Heinlaan et al., 2011). However, Mortimer et al. (2010b) showed that nCuO is 10–20-fold more toxic for *T. thermophila* than bulk CuO mainly due to the released Cu.

The *D. magna* exposed (96 h) to nCuO (<50 nm; 1.97 mg L^{-1}) resulted in significant alterations in (i) encoding protein acting in metal ion binding, (ii) OS, and (iii) exoskeleton (Adam et al., 2015). In all cases, the alteration up-regulated the transcription level of the transcripts genes. Glycogen, lipid and protein concentration in exposed daphnids was lower than unexposed organisms, but when comparing nCuO and metal salt exposures, the microarray evidenced no significant differences in transcribed gene fragments. Thus, the toxicity of nCuO to *D. magna* was caused by Cu ions (Adam et al., 2015). nCuO (26.7 nm; 100 μg L^{-1}) exposure (48 h) led to significant changes in gills transcriptome of adults *D. rerio*, with induction of genes involved in apoptosis, mitogenesis and cell proliferation (Griffitt et al., 2009).

Despite the very limited available data about nCuO ROS generation, cytotoxicity and genotoxicity in for freshwater organisms, it can be concluded that nCuO provoke ROS toxicity mediated by ionic Cu.

5. General remarks and conclusions

Most of the biochemical responses reported are related to the organism's ROS defense mechanisms, mainly through gene expression or changes on anti-oxidant enzymes activities. As mentioned before (Section 3.2), data related with changes in freshwater organism genome and proteome due to the presence of NPs is very scarce. This is indeed surprisingly since omics techniques have already proved a great potential on the recognition of signatures related to specific stress, eventually leading to the discover of new biomarkers. The few data available suggest that the interaction with the organisms is NP-specific. For example, it seems that both $nTiO_2$ and nAg have similar targets in the organism physiology affecting both protein synthesis and circadian regulation, while nZnO and nCuO effects are generally related with OS.

Photoactive NPs such as $nTiO_2$ and nZnO, when under natural or UV light, can generate ROS inducing OS. However, NPs composition can also play an important role on ROS generation, since some metals constituting the NP can instigate Fenton and Weiss-type reactions releasing ROS in the intra or extracellular media. Evidently, and as usually performed, the bioassays should contain a control group exposed to the salt form of the metal constituting the NP, allowing the distinction between the effects provoked by the NP *per se* and/or by the NP dissolution products.

Despite the large number of studies dealing with ecotoxicology of NPs, it is evident that is still not possible to establish crucial predictive structure-activity relationships. This is mainly due to the fact that the majority of the available studies have critical deficiencies on their experimental designs; a comprehensive physicochemical characterization of the particles under the exposure conditions is mostly miscarried or restricted to a secondary task. Despite the scientific community is already aware about the importance of the NPs physicochemical characterization prior and during the bioassay, this is still frequently neglected in the most recent studies giving rise to more confusing and contradictory data. Clearly, this pushes back the possibility to establish a proper environmental risk assessment plan for these current early generations on ENPs (1st and 2nd generation passive and active nanostructures, respectively), while advanced generations of ENPs (3rd and 4th generation nanosystems and molecular nanosystems) may not be far away, bringing additional challenges that require further novel approaches.

The dynamic speciation of the NPs should be assessed in the same exposure media of the bioassay by following key NPs transformations: (i) dissolution, (ii) homo- and heteroaggregation, and (iii) sedimentation. Several analytical tools are nowadays available for the quantification of these physicochemical transformations, each of them having their specific advantages and limitations being able to provide different information on ENPs properties (see reviews Pinheiro and Domingos, 2015; Tiede et al., 2008; Domingos et al., 2009b). This physicochemical characterization approach allows (i) to assess the bioavailable NP-containing species to which the organisms will be exposed, and (ii) to relate the biocompatible or bioadverse effects with the NP-containing species permitting a NP categorization and function. Nanotoxicology is indeed a multidisciplinary field where the study of the NPs physic, chemistry and biological impact is crucial for a complete toxicological assessment. Unfortunately, there is a lack of legislation controlling the production, use and release of these materials to the environment, and new NPs are commercialized every day without an appropriate assessment about their impact in environment and human health. The establishment of national and international laws regulating the production of these materials is mandatory. Furthermore, it is also urgent to increase the number of comprehensive nano(eco) toxicology studies under natural more environmentally-realistic conditions implying the co-presence of ENPs (at low and environmentally-realistic doses) and environmental constituents such as natural organic and inorganic dissolved and colloidal matter. Only with these approaches a comprehensive risk assessment will be possible with production of environmentally safe-by-design ENPs.

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