

## **Section II**

### **Terrestrial and Aquatic Systems**

## 2

## Ecosafety of Nanomaterials in the Aquatic Environment

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### 2.1 Introduction

Nanomaterials (NMs) are novel materials with nanoscale dimensions (1–100 nm) that have attracted increasing attention due to their capacity to alter the physical and chemical properties of conventional materials. They have a wide range of applications into innovative technological uses (nanotechnology). Although a clear definition to characterize these NMs has not been agreed yet, they need to have at least one dimension on the 1–100 nm range. If they have three dimensions within this range, they play a central role in the advance of nanotechnology [1]. Because the definition behind these materials remains controversial, the European Commission defined NMs as “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm” [2].

Since their discovery in the 1980s, NMs production and variety increased enormously, being produced today in many different organic and inorganic chemical forms, such as carbon-based NMs (carbon nanotubes (CNTs) and carbon spheres), metal and metal oxides NMs, quantum dots (QDs), and nanoceramics [3, 4].

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These materials have a wide variety of innovative technological and industrial applications, such as food products, textiles, systems for medical diagnostics (imaging, drug delivery, tumors treatments, and gene therapy), pharmaceuticals, personal care products (PCPs) (cosmetics and sunscreens), renewable energies, sports, electronic devices, telecommunications, paints, self-cleaning surfaces, and environmental applications [5–8]. Recently, a suite of new marine nanotechnologies was developed including antifouling paints (especially containing CNTs) designed to prevent bio-fouling or biofilms on vessel hulls [9, 10] and pollution remediation systems to protect the environment through pollution prevention, treatment, and cleanup of hazardous wastes sites [7, 11] or even in environmental remediation known as *nanoremediation* [7].

These intensive development and applications will produce an increase use of NMs, hence nanowastes will inevitably be released in the aquatic environment and might cause human, ecological, socioeconomics, and environmental impacts, particularly in the marine environment [11]. The uncertainty about their ecosafety and sustainability needs to be urgently addressed [12, 13].

NMs are considered a new class of emerging contaminants, thus there is a pressing need for risk assessment studies in order to introduce ecosafety measures to prevent potential adverse effects to human health and to the aquatic environment.

Due to the need for innovation and potential to synthesize, manipulate, and create, a wide range of nanoproducts/NMs with different chemical compositions, sizes, shapes, coatings, and functional groups are currently being produced. Materials in the nanoform behave quite differently from their bulk counterparts. Their specific physical and chemical characteristics (especially surface and quantum effects) and toxicity make them technologically more attractive [14]. In addition, they can be coated or capped with other chemical compounds to change their chemical properties such as dispersibility and conductivity and to prevent aggregation/agglomeration in aquatic solutions [15]. Due to their surface area/volume ratio even within a single chemical, a higher proportion of atoms are present at their surface, and this increase in surface area partly determines their reactivity. This will result in different chemical reactivity, bioavailability, and ecotoxicity to aquatic organisms, but their impact and ecosafety in the aquatic environment is difficult to predict [11, 16].

In this chapter we review the inputs of NMs in the aquatic environment, the wastewater treatment processes available to remove NMs, the importance of understanding speciation of NMs in the aquatic environment, the toxicological effects on fresh and marine species, the interactive effects of NMs with other contaminants, and the applicability of the existing framework of environmental risk assessment (ERA) to NMs.

## 2.2 Inputs of NMs to the Aquatic Environment

The amount of NMs in the aquatic environment is difficult to predict, so to ensure their ecosafety, proper tools and methodologies for their detection in the aquatic environment need to be developed to accurately assess the ecological risk of these materials [17–19].

Around 50 000 kg/year of nanosized materials are being produced. Some will enter the aquatic environment during the product's life cycle (e.g. by erosion of the materials, accidental spills, or use of products that contain NMs, such as paints, fabrics, and personal health care products), from production facilities and wastewater treatment plants (WWTPs) [12, 20, 21]. As the ultimate sink of most conventional contaminants, the aquatic environment is highly susceptible to NMs exposure either from point (sewage, effluents, and river influx) or from diffuse sources (atmospheric deposition, dumping, and runoff). The first evidence of water contamination of these NMs came from TiO<sub>2</sub> NMs present in runoff water released from self-cleaning exterior facades [22] and in raw sewage and effluents [23]. All these routes allow NMs to disperse through the aquatic environment in quantities that are likely to dramatically increase in the near future but the fate of each of them will vary [24].

## 2.3 How Wastewater Treatment Processes Act in the Removal of Nanomaterials?

The increase use of NMs in consumer products and industrial processes or materials introduces these materials intentionally or unintentionally into the waste streams and in wastewater treatment facilities. The capacity of WWTPs to remove these NMs is controversial. Recent studies suggested that some NMs escape from the wastewater treatment [23, 25, 26]. The fraction of NMs that is not removed has the potential to contaminate surface waters and soils and pose a risk to the biota or affect drinking water resources [27–29]. In addition, sewage sludge applied to agricultural soil may cause exposure of NMs to soil organisms and leach NMs to groundwater [30–33]. Therefore, via wastewater, potential environmental risks are linked to possible bioaccumulation of NMs in natural systems.

The fate of nanosized particles in WWTPs is not well characterized. Wastewater treatment consists of a combination of physical, chemical, and biological processes to remove solids, organic matter, and nutrients from wastewater. Depending on the characteristics of the wastewater and the countries standards, different degrees of treatment can be applied to increase the treatment level (preliminary, primary, secondary, and tertiary and/or advanced treatment, and sometimes disinfection).

However, WWTPs were not designed to remove NMs, thus the study of wastewater processes efficiency has been the focus of current research.

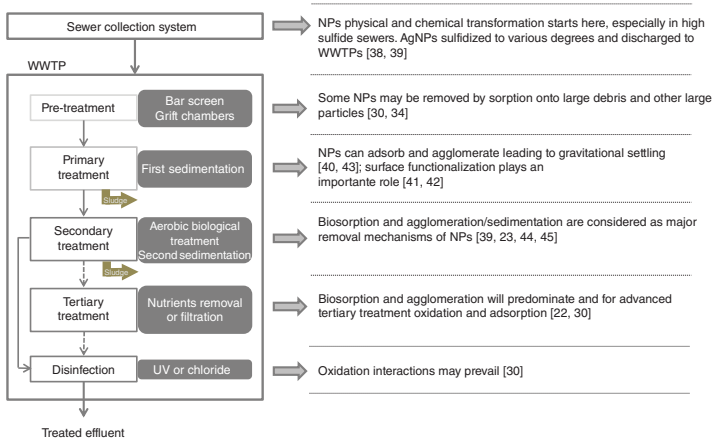
Several studies on NMs removal and release from WWTPs were reported. In Germany, Li et al. [34] concluded that just 5% of AgNPs from the wastewater effluent was discharged rising to low concentrations of AgNPs in treated water (<12 ng/L). In a WWTP effluent containing 100–3000 µg/L of Ti, 10–100 µg/L of Ti still remained in the effluent after treatment [23]. Westerhoff et al. [26] analyzed ten WWTPs with raw sewage whose TiO<sub>x</sub>NPs concentrations ranged from 181 to 1233 µg/L. The WWTP removed more than 96% of the Ti from the influent, and all the effluents had Ti concentrations lower than 25 µg/L. In a pilot WWTP, Ma et al. [35] concluded that both Ag and ZnO NPs were transformed during the WWTP process into Ag<sub>2</sub>S, Zn–Fe oxy/hydroxide, Zn<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, and ZnS, so these compounds appear to be the relevant species for toxicity and fate testing. Similar results for AgNPs were also obtained by Kaegi et al. [36]. These studies showed that WWTPs act as the gateway that control the release of NMs from domestic and/or industrial sources to the aquatic environment via treated effluents discharged into surface waters [34].

## 2.4 So What Is Expected to Occur in WWTPs Processes?

The ability of WWTPs processes to immobilize or destroy NMs depends on the chemical and physical nature of the particles and the residence time in the relevant compartments of the treatment plant. NMs are affected by sorption processes (for example, in primary clarifiers) and chemical reactions [37]. But settling, diffusion, and aggregation according to their size, density, and surface physical chemistry will also affect NPs removal [30]. Figure 2.1 presents a scheme of the NMs route after discharge in the wastewater and evidence of the NMs removal in each phase.

NMs are released to the wastewater collection system where transformation starts. The NMs transformation will influence their subsequent removal in the WWTPs and their bioavailability when released into the aquatic environment. The formation and fate of NMs in wastewater collection systems were investigated by Brunetti et al. [38] and Kaegi et al. [39]. ZnONPs and AgNPs underwent significant transformations during their transport through the sewerage network, being sulfur species the most important endpoint for these NPs [38, 39]. Sulfidation of AgNPs increased with adsorption of humic acids [46] and microbial processes play a direct role in speciation by driving changes in redox conditions and producing materials such as cysteine and histidine [38].

Several researchers investigated the removal of NMs in pre- and primary treatments [30, 34, 40–43] and concluded that sorption and agglomeration are the mechanisms responsible for the NPs removals in these two WWTP phases. NMs



**Figure 2.1** NMs route after discharge in wastewater and removal processes.

can adsorb and agglomerate with other particles present in wastewaters, leading to gravitational settling. Agglomeration involves the adherence of single or cluster of particles into larger masses, due to attractive forces or chemical or mechanical binding. In addition, sorption, agglomeration, and mobility of mineral colloids are strongly affected by pH, ions, and organic matter, so water characteristics may affect sorption and settling of NMs [30, 37]. Li et al. [34] concluded that Ag (ions and NPs) was partly attached to larger particles like suspended organic matter, resulting in removal of approximately 35% AgNPs. In a laboratory study using CeO<sub>2</sub>NPs, the majority of NPs were captured through adhesion to clear sludge, and up to 6 wt% of CeO<sub>2</sub>NPs was found in the exit stream [43]. Hou et al. [40] showed that over 90% of the AgNPs (23 nm) remained in the wastewater even using high suspended solid content during simulated primary clarification experiments. However, NPs surface functionalization plays an important role. King et al. [42] demonstrated that AgNPs (22 nm) coated with a non-ionic stabilizer (PVP) undergo rapid settling in wastewater and will be removed to sewage sludge by hetero-aggregation, rather than continuing to the secondary treatment stage. Results from Jarvie et al. [41] indicated that surface-functionalized SiO<sub>2</sub>NPs (56 nm) were removed by sedimentation to sewage sludge, whereas uncoated SiO<sub>2</sub>NPs continued through the effluent stream. Coated SiO<sub>2</sub>NPs were adsorbed on Tween® molecules and sewage organic matter.

In the biological secondary treatment of WWTPs, where microorganisms are present, NMs can adhere or interact with them, promoting removal by agglomeration and settling of NMs. Interactions between NMs and bacteria appear to involve electrostatic attraction and to be size dependent [47]. NMs removal in activated sludge (A/S) systems seems to be a function of their composition, surface functionalization, size, and zeta potential [23]. In addition, higher biomass (total suspended solids [TSSs]) concentrations improved NMs removal [29, 44]. Gómez-Rivera et al. [45] concluded that CeO<sub>2</sub>NPs (50 nm) were removed in a laboratory scale activated sludge (A/S) system mainly by aggregation and settling, promoted by neutral pH and interactions with organic and/or inorganic wastewater constituents. Batch bioassays demonstrated that CeO<sub>2</sub>NPs only exerted inhibition of O<sub>2</sub> uptake by A/S at concentrations exceeding those in the bioreactor feed (50% inhibition at 950 mg CeO<sub>2</sub>/L). Removals of 39–62% were obtained by Kiser et al. [23] for a group of NMs that includes surface functionalization (citrate-coated Ag, polyvinylpyrrolidone-coated Ag and Au, carboxylate-functionalized Ag, and gum arabic-coated Ag) and of 92–94% for a group of NMs that has properties that favor association with fresh activated sludge (nonfunctionalized fullerene, carboxylate- and sulfate-functionalized yellow-green fluorescent microspheres, and tannic acid-capped nanogold (AuNPs)). These authors concluded that zeta potential or size may not be sufficient for making *a priori* estimates of the removal of NMs in fresh activated

sludge. Similar results were obtained by Kaegi et al. [39] for AgNPs (up to 10 nm) and AuNPs (up to 10 nm), where removals of approximately 99% were observed irrespective of size, coating, or type (elemental composition) of the core material by attachment to sludge flocs (hetero-aggregation).

Finally, in the tertiary treatment, the removal of the portion of the remaining organic and inorganic solids and pathogenic microorganisms occurs through a filtration step and/or the removal of nutrients. This treatment may be followed by chemical disinfection. The impact of this treatment in the removal of NMs is still not well addressed. A study was conducted in a WWTP in central Arizona (USA) that uses activated sludge process and tertiary filtration to treat a raw sewage containing 100–nearly 3000  $\mu\text{g Ti/L}$  [23]. Results showed Ti concentrations between secondary and tertiary effluents of 20 and  $12 \pm 2 \mu\text{g/L}$ , respectively, and the same concentration (36  $\mu\text{g/L}$ ) for non-filtered samples. These authors concluded that the majority of Ti is associated with solids in wastewater, so as tertiary effluent has low solid levels (4 mg/L TSS), removals were higher in previous WWTP phases. Brar et al. [30] considered that in these phases (tertiary and disinfection), the NMs removal processes previously discussed would predominate. However, there are many studies on the effect of NMs on the removal of nitrogen and phosphorus and on the biological communities responsible for nutrient removal. It seems that NMs have a negative impact on the removal of these nutrients from wastewater, especially for high NMs concentrations [48–51].

As a result of the different treatment phases, sludge from primary and secondary settling must be digested and dewatered and sent to landfill or reused for agricultural application. However, some of the NMs will persist under various biosolids processing procedures [35, 52]. Therefore, the fate and ecosafety of NMs in soils and water and interactions with plants coming for threat WWTPs sludge should be carefully evaluated.

## 2.5 The Importance of Understanding Speciation of NMs

Trace metal toxicity in natural systems is difficult to decipher due to the variety of metal ions and metal complexing agents present in the matrix. Thus, it is fundamental to know the specific dynamic metal speciation, also termed chemodynamics [53] to understand their toxicity. From the physicochemical point of view, the traditional approach is to simplify the problem considering that only the free metal ion (the hydrated cation) is toxic to the organisms, i.e. the well-known Free Ion Activity Model (FIAM) [54] and its evolution to the Biotic Ligand Model (BLM) [55].



The introduction of metal containing NMs adds a level of complexity to these already challenging studies. One must always consider the dissolution of NMs functioning as a source of metal ions [56], as well as the possibility of exchange of metal in the particle by another metal previously in solution and the interaction of the metal ions with the charged polymer layer often used to stabilize metal containing NMs [57]. In this context it is fundamental to quantify the amount of metal in the NMs and the amount of metal in solution during an exposure experiment.

The measurement of total metal in solution, comprising the metal contained in the NMs, the free metal ion, and the metal complexed by the matrix ligands is routinely performed by spectroscopic techniques, normally by inductively coupled plasma-mass spectrometry (ICP-MS).

The quantification of the concentration and/or number of NMs in solution is an involved problem normally requiring the physical separation of the NMs from the dissolved components of the matrix, usually by ultrafiltration [58], dialysis [59], or ultracentrifugation [60], followed by metal quantification by ICP-MS. Recently, the development of single particle ICP-MS [61, 62] provides a powerful, albeit expensive, technique to quantify very low NMs concentrations in complex matrices without previous separation.

Regarding the free metal ion activity, the traditional approach in ecotoxicological studies is not to measure but rather to buffer the free metal using a large total metal concentration and a metal buffer chelating agent (like EDTA). In experiments involving metal containing NMs, this approach is impossible since the chelating agent will affect the stability of the NMs and the metal speciation in the matrix [63]. The absence of metal buffer and the uncertainty in the amounts of free metal ions and metal in the NM makes it necessary to directly measure the free metal ion activity.

The quantification of free metal ion activity in solution, in principle, is not affected by the presence of NMs; nevertheless for the environmentally relevant concentrations, there are few available techniques [64], being the most adequate is the Donnan membrane technique (DMT) [65, 66] and the absence of gradients and Nernstian equilibrium stripping technique (AGNES) [67].

DMT is based on the transport of metal ions from solution, through a negatively charged semipermeable cation exchange membrane, to an acceptor solution. It has the advantage of being able to perform simultaneous measurements of several elements. When the solution is added, the cations will permeate the membrane until equilibrium is attained (usually 24–48 h), whereas the transport of anionic species is blocked by the negative charge of the membrane. The measurement of the total metal in the acceptor after reaching equilibrium yields the free metal ion concentration. The membranes used are calcium-based, implying that the background electrolyte will have calcium ions, which can be a major disadvantage if the matrix is calcium poor.

DMT is probably the best technique to determine several free metal ions in solution as long as the matrixes have already significant calcium content and were extensively applied to quantify free metal ions in soils [68, 69], natural waters [70–72], and ecotoxicological assessment [73–75]. In studies involving NMs, DMT was used to study the behavior of capped (by an organic coating composed of a mixture of C12–C15 alkyl benzoate, polyhydroxystearic acid, and triethoxycaprylsilane) and uncapped ZnO (17.5 and 21 nm) in synthetic seawater [76] and the UV-induced photochemical transformations of citrate-capped AgNPs (20, 40, 60, and 80 nm) suspensions [77].

AGNES is an electrochemical stripping technique designed to quantify low free metal ion concentrations. It consists of two steps: a deposition step (where the metal ion is amalgamated in the working mercury electrode) and a stripping step (where the deposited metal is reoxidized and quantified). The major drawback of AGNES is the limited number of metals that it can study (Zn, Cd, Pb, Cu) when using a mercury electrode, but recently its extension to solid electrodes [78] opens good perspectives for its application to other metal ions. AGNES has been used in NPs studies involving the dissolution of CdTe/CdS QDs (3–4 nm) [63], the dissolution kinetics and solubility of ZnONPs (6, 20, and 71 nm) [79], the toxicity of ZnONPs (20–40 nm by TEM and 66.5–153.1 by DLS in water) on *Daphnia magna* [80], the bioavailability of Cd in presence of n-TiO<sub>2</sub> (5–20 nm) to *Corbicula fluminea* [81], and the toxic kinetics and tissue distribution of COOH-CdTe QDs (6 nm) in *Mytilus galloprovincialis* [82].

Another significant difference in the NMs' case is the aggregation/disaggregation phenomena that might happen when introducing the NMs in natural systems due to the changes of matrix conditions (like pH and ionic strength) and their interaction with other components of the matrix, like the natural organic matter (NOM) [83, 84].

The fate of NMs in the environment has been an object of great interest and many studies have been published [85], and it is important that this knowledge is used to understand the changes to the NMs and their interaction with the metal ions in solution during an ecotoxicological assessment, since they affect the solubility of the NMs and their ability to bind metal ions in solution.

Techniques and recommendations on how to follow the physicochemical changes of NMs in solution are abundant in the literature and many analytical techniques are accessible for size measurements comprising microscopy techniques, dynamic light scattering (DLS), fluorescence correlation spectroscopy (FCS), nanoparticle tracking analysis (NTA), and separation-based techniques, such as field flow fractionation (FFF), hydrodynamic chromatography (HDC), or analytical ultracentrifugation [86–88].

Within this framework it is important to recognize that an ecotoxicological assessment using NMs cannot be performed under the same hypothesis as for

metal ions. The design and execution of an environmentally relevant toxicological experiment should take into consideration the following aspects [89]:

- (a) In the exposure solution the physicochemical conditions of the medium should emulate those of the environmental matrix, and the following parameters need to be known and/or changed in a controlled way: temperature, pH, ionic strength, light, concentration of other metals, and complexing agents (especially NOM).
- (b) In the ecotoxicological experiments in addition to the control and the metal-based NM contaminated solution, it is necessary to perform an additional control containing the same total metal concentration added in soluble form. To be able to analyze the toxicity results, it is necessary to follow, in the exposure media during the time of the experiment, the (i) metal containing NMs dissolution and aggregation/disaggregation behavior, (ii) free metal concentration, and (iii) metal containing NMs interaction with the other components of the matrix.

The ecotoxicity modeling of metal containing NMs also presents some interesting challenges, not only due to the possibility of direct internalization of NMs but also due to the possible dissolution of NMs and corresponding temporal variation of both total dissolved and free metal ion in solution. Therefore, the use of BLM becomes impossible since one of its main assumptions is that both total dissolved metal and free metal are constant during exposure.

Another interesting development in terms of modeling is the application of dynamic energy budget (DEB) theory to understand NP toxicity, namely, CdSe QDs (5 nm) toxicity [90, 91] and ZnONPs (20–30 nm) to *M. galloprovincialis* [92].

The ideal development would be the integration of physicochemical bioavailability models, considering the possible direct internalization of the NMs and their fate inside the organism, with the DEB model to provide a realistic modeling of the effect of NMs in the environment.

## 2.6 Ecotoxicological Effects of NMs in Freshwater Organisms

Most of the assessment of ecotoxicological effects of NMs in the aquatic environment was mainly focused on four out of five classes of NMs based on their chemical composition: carbon-based NMs (e.g. CNTs and fullerenes) [93, 94]; metal NPs and metal-oxide NPs (e.g. TiO<sub>2</sub>NPs, AgNPs, and CuONPs) (e.g. [95–101]); and QDs [102]. Results so far suggested that NMs can be toxic to freshwater organisms, and effects are particle-type dependent. Furthermore, data from the field is scarce or inexistent in the aquatic environment.

Yan et al. [103] showed that the exposure to hydroxylated (OH-MWCNT;  $4 \pm 1.5$  nm; 0.5 mg/L) and carboxylated multi-wall (COOH-MWCNTs;  $4 \pm 1.5$  nm; 0.5 mg/L) for 4 and 18 days induced more stress to the fish *Carassius auratus* than raw MWCNTs ( $12 \pm 2$  nm; 0.5 mg/L). Furthermore, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), OH-MWCNT, and COOH-MWCNTs enhanced Cd toxicity to *D. magna* [94], suggesting that functionalization plays an important role in nanotoxicity. The exposure to SiO<sub>2</sub>NPs (80–100 nm; 1–5 mg/L; 96 h) altered plasma electrolytes (Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup>) levels, hematological parameters (e.g. hemoglobin and red and white blood cells), and Na<sup>+</sup>/K<sup>+</sup> ATPase activity in gills of the teleost fish *Labeo rohita* [104].

The exposure of several algal species, such as *Scenedesmus quadricauda*, *Chlamydomonas moewusii*, and *Chlorella vulgaris*, to TiO<sub>2</sub>NPs (27 nm; up to 300 ppm, 22 days) led to a decrease in their growth and metabolic functions [105]. The toxicity of TiO<sub>2</sub>NPs was higher to the freshwater algae *Pseudokirchneriella subcapitata* followed by *Ceriodaphnia dubia* and fathead minnow *Pimephales promelas* [106] and induced genotoxicity and cytotoxicity in *C. dubia* [107]. The increased bioavailability of metal ions with the decrease in particle size contributed to explain the toxicity levels of each metal NP [98, 108, 109]. Several metal NPs are reported to be toxic to the microalgae *P. subcapitata*, and EC<sub>50</sub> (72 h) indicated that toxicity decreased as follows: ZnONPs (50–70 nm) > CuONPs (30 nm) > TiO<sub>2</sub>NPs (25–70 nm) [109]. Zhu et al. [110] showed that the chronic exposure to TiO<sub>2</sub>NPs (100 nm; up to 50 mg/L; 96 h) resulted in bioaccumulation of NPs interfering with the daphnid feeding and inhibiting its growth and reproduction. In a short-term exposure, no lethal effects of TiO<sub>2</sub> NPs (approximately 24 and 210 nm; 150 mg/L; 5 days) were found on the fish *Poecilia reticulata* although NPs were able to penetrate into the fish blood through the gills and intestine [111]. The toxicity of TiO<sub>2</sub>NPs (approximately 24 nm; up to 1.0 mg/L; 14 days) to the rainbow trout was associated with oxidative stress, organ pathologies, and the induction of antioxidant defenses [112]. Dietary exposure of this fish species to TiO<sub>2</sub>-NPs (<100 nm; 100 mg/kg; 8 weeks) caused changes in Cu and Zn levels in the brain, with biochemical alterations in the gills and gut [113]. TiO<sub>2</sub>NPs were reported to decrease the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the gills and intestine, alteration of total glutathione levels (e.g. gills and liver) and the presence of apoptotic bodies in some hepatocytes of the rainbow trout [112]. Dietary exposure of rainbow trout to TiO<sub>2</sub>NPs (100 nm; 100 mg/kg; 8 weeks) reduced 50% of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the brain and thiobarbituric acid reactive substances (TBARS) in the gill and intestine, while Ti accumulation was detected in several fish organs (gills, gut, liver, brain, and spleen) [113]. TiO<sub>2</sub>NPs (20 nm; 5.3 mg/L; 48 h) under natural UV radiation were toxic to aquatic microbes and generation of ROS and cell membrane damage were observed [114].

CeO<sub>2</sub>NPs (15 and 30 nm; 1 mg/L; 96 h) and SiO<sub>2</sub>NPs (7 and 10 nm; 1 mg/L; 96 h) induced toxicity against *D. magna* and to the larvae of *Chironomus riparius* [115]. CeO<sub>2</sub>NPs promoted DNA strand breaks in both species, whereas neither exposure to SiO<sub>2</sub> nor TiO<sub>2</sub> had a genotoxic effect on either species. Moreover, the DNA damage was directly related with the mortality of *C. riparius* exposed to CeO<sub>2</sub>NPs [115].

Lethal effects after 24 h of exposure to CuONPs (30 nm) and ZnONPs (70 nm) were reported against *Daphnia* and *Tetrahymena thermophila*, and the toxicity was mainly attributed to the solubilized ions [116, 117]. However, ionic metals released from metal NPs (TiO<sub>2</sub>, 25–70 nm; ZnO, 50–70 nm; CuO, 30 nm) only partly explain the nanotoxicity against the yeast *Saccharomyces cerevisiae* [118]. Chronic exposure of the gastropod *Potamopyrgus antipodarum* to CuONPs for 9 weeks resulted in the accumulation of Cu and their toxicity varied with NP shape: rod (8 nm width, approximately 40 nm length), spheres (approximately 7 nm), or platelet (approximately 1 μm length, approximately 270 nm width) [119].

AgNPs (44 nm; 0.3 mg/L) and CuONPs (33 nm; 10 mg/L) showed bactericidal effects, while ZnONPs (<1000 nm; 10 mg/L) showed bacteriostatic effects against *Pseudomonas putida* after 60 min exposure [120]. In lake waters, the dissolution of ZnONPs (4.5 and 27 nm), carbon-coated CuNPs (7.5 and 27 nm), and CuONPs (7.5 and 45 nm) was higher than that of AgNPs (10 and 100 nm) and increase with the decrease of pH, ionic strength, and dissolved organic matter [56]. The latter can stabilize AgNPs, while sunlight can induce the transformation of AgNPs by causing particle-fusion or self-assembly to form aggregation of NPs in aquatic environment [121], indicating that the effects of NPs in aquatic environments depend also on light availability.

The exposure to CuONPs (30 nm; up to 175 mg/L; up to 48 h) led to ultra-structural changes in the midgut epithelium of daphnids and the presence of NPs in circular structures, analogous to membrane vesicles from holocrine secretion [122].

Exposure to sublethal concentrations of CuONPs (<50 nm; up to 1000 mg/L; 10 days) led to adverse effects on the growth and feeding behavior of invertebrate detritivore *Allogamus ligonifer* [101]; however, the effects on microbes [123] and invertebrate detritivores [124] decreased with the increase in NP size and presence of NOM. Histological and biochemical analyses revealed that the gills of zebrafish could be the primary target for CuNPs [125]. CuNPs decreased gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity up to 58%, and the transcription of stress response genes increased, such as the hypoxia-inducible factor 1, heat-shock protein, and Cu transport regulatory protein [125]. In fungal populations, CuONPs (<50 nm; up to 200 mg/L; up to 10 days) induced oxidative stress and increased the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and the extracellular enzyme laccases, which requires Cu as cofactor [126]. Also, CuONPs (12, 50, and 80 nm; up to 100 mg/L; 5 days) led to plasma membrane disruption,

intracellular accumulation of ROS, and DNA strand breaks in aquatic fungi [126]. Waterborne and dietborne exposures of the snail *Lymnaea stagnalis* to ( $^{65}\text{CuO}$ )NPs (17 nm; up to 72 nM; 24 h) resulted in the bioaccumulation of the NPs most probably due to NP uptake [127].

The toxicity of AgNPs (20, 40, and 100 nm; 10  $\mu\text{M}$ ; 72 h) in *Synechococcus* sp. is explained by both the NP form and ionic Ag released by dissolution from the NPs [128]. Moreover, the exposure to AgNPs (5–25 nm; 48 h) and the ionic counterpart led to similar effects on mortality and abnormal swimming of *D. magna* [129]. The toxicity of AgNPs (50 nm; up to 10 mg/L; 24 h) in microalgae *C. vulgaris* was associated with a strong decrease in chlorophyll content and algal cell density, increased intracellular accumulation of reactive oxygen species (ROS), and lipid peroxidation (LPO) indicating that NPs induced oxidative stress [130]. The exposure of the mussel *Elliptio complanata* to AgNPs (20–80 nm; 0.8–20  $\mu\text{g/L}$ ; 24 h) increased the levels of MTs and LPO and induced changes in the protein ubiquitin, and DNA strand breaks in the digestive gland [131]. Because effects promoted by  $\text{Ag}^+$  differed from those of AgNPs, a significant role in the toxicity was attributed to the NP form [131]. Results were consistent with those found when examining the proteomic profiles of *D. magna* exposed to AgNPs (3.0 and 11 nm; 20 mg/L; 6 and 10 months) and  $\text{Ag}^+$  that showed an increase of carbonyl and hemoglobin levels only in the presence of AgNPs [132].

AgNP exposure (42 nm; up to 166  $\mu\text{g/L}$ ; 120 h) resulted in lethal and sublethal effects in zebrafish with various phenotypic deformities, including distorted yolk sac, degenerated body parts, twisted notochord, impaired blood circulation, low heart rate, and pericardial oedema [133]. AgNPs (5–20 nm; 100  $\mu\text{g/L}$ ; 72 h) induced apoptosis or programmed cell death in zebrafish embryos [134]. The exposure of the fish *Japanese medaka* to AgNPs (49.6 nm; up to 25  $\mu\text{g/L}$ ; up 10 days) led to DNA damage, oxidative stress, and upregulation of genes related to metal detoxification, metabolic regulation, and free radical scavenging activity [135]. AgNPs (5–20 nm; up to 100  $\mu\text{g/mL}$ ; 72 h) reduce hatching of zebrafish embryos [134] potentially compromising fish populations at long time.

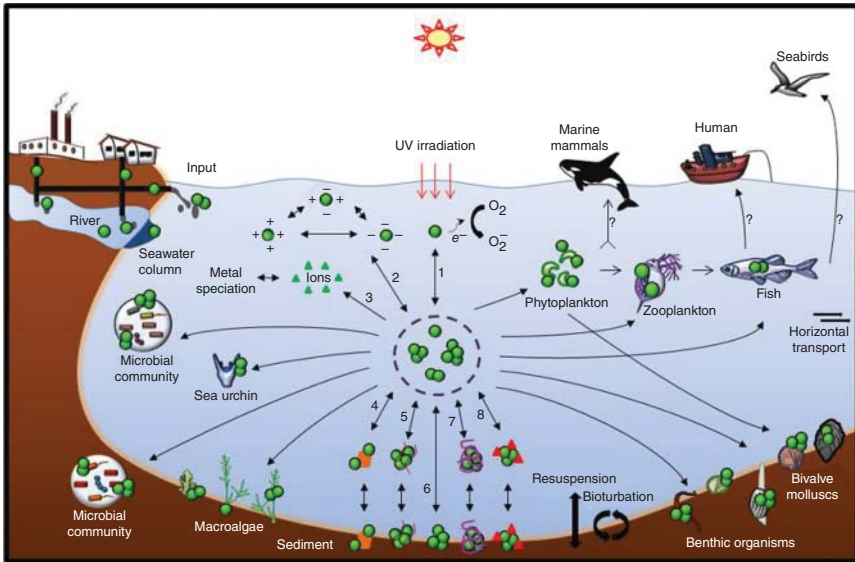
Lethal toxicity of metal NPs is generally higher for organisms at lower trophic levels (filter-feeding invertebrates) compared with higher trophic levels (e.g. secondary consumers). Despite the few information available on NP effects at the level of communities and ecosystem processes, the knowledge on structural and functional responses of aquatic biota to NPs is required to predict the risk of these NMs. Most studies were done on freshwater bacterial communities or periphyton. For instance, the short-term exposure to AgNPs (<5 nm; 12.5 and 100  $\mu\text{M}$ ; 2 h) inhibited the respiration and photosynthesis and the activities of extracellular enzymes of periphyton [136]. However, phosphorus interactions with AgNPs attenuated the effects of NPs on the growth and stoichiometry of periphyton [137]. On the other hand, the extracellular polymeric substrate of

periphytic communities altered the stability and surface of 25 nm of AgNPs and CeO<sub>2</sub>NPs [138]. The exposure of benthic bacterial communities to TiO<sub>2</sub>NPs (22.9 nm; 1 mg/L; up to 30 days) resulted in a decrease in bacterial abundance in artificial stream sediments [139]. TiO<sub>2</sub>NPs led to a decrease in planktonic bacterial diversity and to an increase in sessile bacterial diversity [140]. TiO<sub>2</sub>NPs (10–20 nm; 5.3 mg/L; up to 48 h) were toxic to planktonic and biofilm communities under natural levels of UV radiation and low TiO<sub>2</sub> concentrations, and effects were explained by the generation of ROS [114]. However, the exposure to low concentrations of AgNPs (<100 nm; up to 1000 µg/L; up to 30 days) induced minor changes in genetic diversity of bacterial communities from estuarine sediments [141]. Metal NPs (Ag and CuO <100 nm) led to changes in the structure and activity of fungal and bacterial decomposer communities [100, 142] with impacts to plant litter decomposition in streams [100].

## 2.7 Ecotoxicological Effects of NMs in Marine Organisms

NMs behave differently in fresh and marine systems. In seawater, NMs undergo complex interactions and transformations (e.g. aggregation/agglomeration, dissolution, NOM stabilization) [143–145], so their chemical nature and behavior is crucial to assess their bioavailability, bioaccumulation, and toxicity to marine organisms [145]. Aggregation/agglomeration kinetics, changes of the surface charge, and sedimentation rates of commercial NMs are salinity/ionic strength and pH dependent, as reported for CuONPs (33 nm) [146] and COOH-CdTe QDs (6 nm) [82, 102]. In aqueous medium, the ionized carboxylic groups confer negative surface charge for CdTe QDs aggregates, but salinity increase induces lower negative surface charge, higher hydrodynamic diameter ( $d_h$ ), and sedimentation rate of QDs in seawater when compared with Milli-Q water, confirming the tendency of commercial NMs to aggregate/agglomerate in seawater, specially due to low repulsive electrostatic forces [102].

Therefore, ecotoxicological effects of NMs in seawater are different from freshwater especially because in seawater agglomeration/aggregation, dissolution and precipitation affect their bioavailability and ecotoxicity [147]. Figure 2.2 shows the different sources and interactions of NMs to the marine environment. Behavior and fate of NMs in marine environment are highly dependent on environmental conditions (e.g. ionic strength, pH, temperature, presence of NOM, and suspended particles), as well as on their nano-specific properties (e.g. size, shape, particle surface charge, functionalization, and coating) [144, 146, 148]. The interaction of NMs with surrounding components also affects their fate in seawater and sediments and



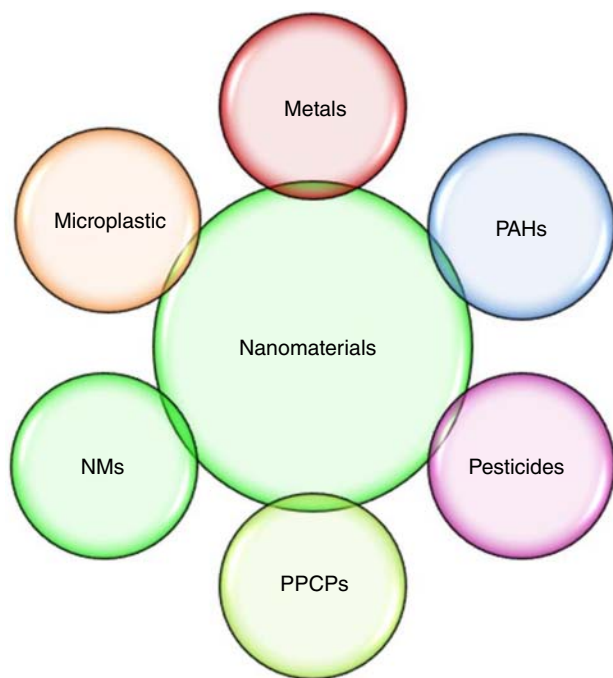
**Figure 2.2** General scheme illustrating the potential pathways for the behavior and fate of nanomaterials (NMs) in the marine environment and their biological interactions at different trophic levels: (1) photo-oxidation, (2) surface charge change, (3) dissolution, (4) adsorption to suspended particles, (5) NOM stabilization, (6) sedimentation, (7) adsorption to biomolecules, and (8) interaction with other contaminants (*Trojan horse* effect).

thus determines their biokinetics and toxicity in benthic and pelagic organisms (Figure 2.2).

In natural environmental scenarios, different states of NMs may occur and interact with marine organisms: individual NMs; homo-aggregates; individual and NMs aggregates/agglomerates stabilized by NOM; individual and NMs aggregates adsorbed to suspended particle and/or biomolecules (e.g. clays, exopolymers and mucus); and individual and NMs aggregates adsorbed to organisms (e.g. phytoplankton and zooplankton), which interact with other organic and inorganic contaminants, as well as metal with ions released from metal-based NMs, metal complex formed after NM dissolution and ROS formed by NMs photo-activation (i.e.  $O_2^-$  and  $OH^-$ ) [11, 145, 148, 149] (Figure 2.3).

The literature about the impact of NMs on aquatic environment increased since 2006, when the first nanoecotoxicological research was published [28]. Since then, a considerable effort was made by the scientific community to assess several ecotoxicological end points to quantify sublethal effects in marine organisms exposed to different classes of NMs, such as inorganic and organic NMs (reviewed in [145, 150–153]). Even so, there is more information regarding





**Figure 2.3** Scheme illustrating the potential interaction of NMs with other NMs and different types of pollutant in the aquatic environment. PAHs: polycyclic aromatic hydrocarbons; PPCPs: pharmaceutical and personal care products.

the effects of NMs in freshwater species when compared with seawater ones, especially for  $\text{TiO}_2$  NPs, metal, and metal oxides NMs, while for ecotoxicity tests using bivalve species, more information exist on seawater species compared with freshwater ones [145]. Among marine invertebrates, bivalve mollusks were the most studied group (about 50%), followed by polychaetes and echinoderms, while sediment-dwelling invertebrates were less studied (about 20%) (reviewed by [153]). Among marine species, the blue mussel *Mytilus* spp. is the biological model most used to assess the effects and mode of action (MoA) of NMs in the marine environment [145]. Based on laboratory experiments there is information regarding NPs accumulation and ecotoxicological effects and MoA in marine organisms [144, 150–152]. Adsorption and accumulation of NMs were reported for a wide array of marine organisms at different trophic levels, such as bacteria, phytoplankton, zooplankton, macroalgae, echinoderm, polychaete, bivalve mollusks, and fish (Figure 2.2), while less information is available for marine mammals and seabirds [152, 154–157]. Furthermore, sea urchin was recently

indicated as a suitable model for evaluating the effects of NMs on the early life stage and on the immune system [153, 158].

Despite NMs accumulation and trophic transfer in reconstructed marine trophic chain, limited biomagnification was confirmed, indicating the urgent need to assess the ecotoxicological effects of NMs in environmental relevant conditions, such as mesocosms and multispecies exposures (reviewed in [159]). Furthermore, to assess the ecosafety of NMs, further studies are needed to investigate the possible impact on human health due to the consumption of NMs-contaminated seafood.

Differential bioaccumulation and tissue-specific responses were detected in marine invertebrate tissues after exposure to NMs, such as in oysters *Crassostrea virginica* exposed to AgNPs (20–30 nm; 0.02–20 µg/L; 48 h; [160]), marine mussels *M. galloprovincialis* exposed to CuONPs (31 nm; 10 µg/L; 15 days; [96]), AgNPs (42 nm; 10 µg/L; 15 days; [161]), and CdTe QDs (6 nm; 10 µg/L; 15 days; [78]). Inorganic NPs (e.g. 42 nm Ag, 10–100 nm CuO, 22 nm TiO<sub>2</sub>, and 6 nm QDs) are accumulated by mussel gills, translocated to the hemolymph and transported to the digestive gland where it induces changes in lysosomal membrane stability (LMS) and antioxidant enzymes activities, immunotoxicity, neurotoxicity, genotoxicity and pre-apoptotic processes [82, 95–97, 102, 162, 163], suggesting that *in vitro* and *in vivo* exposure support the hypothesis that several toxic effects of NMs on marine organisms are directly or indirectly mediated by oxidative stress. However, mechanisms of NMs-mediated toxicity are not well established and depend on nano-specific properties, exposure conditions, species, tissue, and end point analyzed.

Oxidative stress, changes in membrane proteins, and DNA damage are the main MoA of NMs in bivalve tissues (reviewed in [145]), while toxic effects of metal-based NMs were also related to extra- and intracellular dissolution and release of metal ions from NMs (reviewed in [152]). Alterations of antioxidant enzymes activities, ROS generation, and oxidative damage induced by different QDs were observed in the marine diatom *Phaeodactylum tricorutum* exposed to CdSe and CdSe/ZnS QDs (3.2–3.5 nm; 72 h; [164]), polychaete *Hediste diversicolor* exposed to CdSe/ZnS QDs (5.9 nm; 0.001–1 ng/(g w. sediment; [165]), marine mussel *M. galloprovincialis* exposed to CdTe QDs (6 nm; 10 µg/L; 14 days; [82, 102]), and fish *Gasterosteus aculeatus* exposed to CdS QDs (4.2 nm; 5–500 µg/L; 21 days, [166]) and showed that the modification of NM surface charges, functionalization, and coating have an effect on their uptake, accumulation and toxicity in marine organisms, indicating that the progress in the synthesis and modification of NM surface contribute to greater ecosafety of NMs in the aquatic environment.

Moreover, early life stages are a sensitive target for metal-based NMs. Embryo development is an important target for different types of NMs due to metal ion

solubilization. Toxicity may be related to other mechanisms that are particle specific as the formation of a NM protein corona that might affect uptake routes and toxicity in different types of embryos [153].

## 2.8 Interactive Effects of NMs with Other Contaminants

Ecosafety and risk assessment of NMs in the marine environment should consider not only their inherent nanotoxicity but also the potential interaction of NMs with other pre-existing contaminants [167]. According to the *Trojan horse* concept, NMs can act as a carrier for other contaminants, which can change the bioavailability, uptake, accumulation, and toxicity of both NMs and contaminants in marine organisms [168].

In Figure 2.3 the possible interactions of NMs with other contaminants in the marine environment are described. Although the physicochemical interactions of NMs with several contaminants were investigated, the interactive effects of NMs with other contaminants in marine organisms are scarce. Regarding species, bivalve mollusks were the most studied group (64.3%) (with the mussel *M. galloprovincialis* as the main biological model used (50%)), while fish and sea urchin represent 28.6 (the marine fish *Dicentrarchus labrax* (21.4%)) and 7.1%, respectively (Table 2.1). They only exist for TiO<sub>2</sub>NPs (71.4%), AuNPs (14.3%), C<sub>60</sub> (7.1%), and SWCNTs (7.1%), while Cd<sup>2+</sup> (28.6%) and 2,3,7,8-tetrachlorodibenzo-p-dioxins (TCDD; 28.6%) were contaminants whose interaction with NMs were the most investigated, followed by polycyclic aromatic hydrocarbons (PAHs: benzo(a)pyrene, fluoranthene, and phenanthrene), pro-oxidant compound menadione, and two emergent contaminants (1–5 μm microplastics and 15 nm AgNPs) (Table 2.1). TCDD accumulation increased in whole soft tissues of mussels *M. galloprovincialis* exposed to a mixture of TiO<sub>2</sub>NPs (27 nm; 100 μg/L) and TCDD (0.25 μg/L) for 96 h [173], but a decreased in TCDD accumulation occurred in the digestive gland [175], indicating that the *T. horse* effect in marine mussels is tissue dependent, such as observed for the exposure to NMs alone [145, 160]. On the other hand, no Cd accumulation occurred in the digestive gland of *M. galloprovincialis* after the co-exposure to TiO<sub>2</sub> NPs (27 nm; 100 μg/L) and Cd<sup>2+</sup> (100 μg/L) [172], but TiO<sub>2</sub> NPs accumulation increased in the gills [155]. NPs-induced chromosomal damage and partial recovery of genome template stability in the marine fish *D. labrax* and mussel *M. galloprovincialis* after co-exposure to TiO<sub>2</sub>NPs (24 nm; 100 μg/L) and Cd (100 μg/L) [155, 177], indicating that changes in the NMs behavior during co-exposure lead to a decrease on nano-specific toxicity. Reduced C<sub>60</sub> accumulation was observed in *M. galloprovincialis* after the co-exposure to C<sub>60</sub> (100–200 nm; 0.1–1 mg/L) and

**Table 2.1** Interactive effects of nanomaterials (NMs) with other contaminants in marine organisms.

Species	NM			Pollutant			Cell/ tissue <sup>b)</sup>	Interactive effects <sup>d)</sup>	References
	Type <sup>a)</sup>	Size (nm)	Concentration (µg/L)	Type <sup>a)</sup>	Concentration (µg/L)	Exposure time			
Bivalve mollusks									
<i>Mytilus edulis</i>	TiO <sub>2</sub>	62	200, 2000	Benzo(a) pyrene	20	96 h	DG, H, W	Decreased B(a)P accumulation. No effects on SOD and GPx. Increased chromosomal damage and CAT	Farkas et al. [169]
<i>Mytilus galloprovincialis</i>	Au	15.6	750	Menadione	1 mM	24 h	DG	Reduced Au accumulation. No effects on oxidative stress parameters (TrxR; GSH/GSSG ratio; protein thiol group)	Tedesco et al. [170]
	C <sub>60</sub>	100-200	100, 1000	Fluoranthene	32	72 h	DG, G, H, M	Reduced C <sub>60</sub> accumulation. Additive (DNA damage) and synergic effects (GSH content)	Al-Subiai et al. [171]
	TiO <sub>2</sub>	27	100	Cd	100	96 h	DG, E, H	No effect on Cd accumulation. Synergic effects (lysozyme, TLR-i genes). Decreased retarded development induced by Cd <sup>2+</sup>	Balbi et al. [172]
	TiO <sub>2</sub>	27	100	TCDD	0.25	96 h	DG, G, H	Increased TCDD accumulation. Antagonistic (hemocyte phagocytosis, ABC transport) more than synergic effects (neutral lipid content and lysosome/cytoplasm volume ratio, transcription of estrogens receptors)	Canesi et al. [173]

(Continued)

Table 2.1 (Continued)

Species	NM			Pollutant			Cell/ tissue <sup>b)</sup>	Interactive effects <sup>d)</sup>	References
	Type <sup>a)</sup>	Size (nm)	Concentration ( $\mu\text{g/L}$ )	Type <sup>a)</sup>	Concentration ( $\mu\text{g/L}$ )	Exposure time			
	TiO <sub>2</sub>	24	100	Cd	100	96 h	G	Decreased Cd accumulation. Antagonistic ( <i>abcb</i> 1 gene, GST, DNA damage) more than additive effects (NO production).	Della Torre et al. [174]
	TiO <sub>2</sub>	24	100	Cd	100	96 h		No effect on chromosomal damage. Partial recovery of genome template stability	Rocco et al. [13]
	TiO <sub>2</sub>	27	100	TCDD	0.25	96 h	DG	Decreased TCDD accumulation. Up regulated 28 DEGs and down-regulated 34 DEGs. Decreased tissue damage	Banni et al. [175]
<i>Scapharca subcrenata</i>	TiO <sub>2</sub>	$\leq 10$	500	Phenanthrene	20.9 ng	35 d	G, M, PF	Increased phenanthrene accumulation	Tian et al. [176]
Fish <i>Dicentrarchus labrax</i>	TiO <sub>2</sub>	27	1000	TCDD	46 pg/L	7 d	L, G, Er, S, M	No effect on TCDD accumulation	Della Torre et al. [174]

	TiO <sub>2</sub>	24	1000	Cd	100	7 d	Er, M	Decreased NPs induced chromosomal damage. Partial recovery of genome template stability	Nigro et al. [177]
	TiO <sub>2</sub>	27	1000	TCDD	46 pg/L	7 d	L	Down-regulated <i>ahrr</i> , <i>erf2</i> , <i>Abcb1</i> , and <i>Abcb2</i> genes. No effects on <i>Cyp1a</i> , <i>gst</i> , and <i>elmod2</i> genes	Vannuccini et al. [178]
<i>Pomatoschistus microps</i>	Au	5	200	Microplastic (1–5µm)	184	96	He, O	No effects on NP accumulation and NP-induced toxic effects	Ferreira et al. [179]
Sea urchin									
<i>Strongylocentrotus droebachiensis</i>	COOH-SWCNT	1.4 µm × 2–20 nm	200–5000	PAAM-Ag NPs (15 nm)	20–500	96	E	Decreased toxic effects on early development stages	Magesky and Pelletier [180]

- a) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxins (TCDD); poly(allylamine)-coated silver nanoparticles (PAAM-AgNPs); single-walled carbon nanotubes (SWCNT).
- b) Digestive gland (DG); embryo (E); erythrocytes (Er); gills (G); head (He); hemolymph/hemocyste (H); liver (L); muscle (M); pseudofeces (Pf); skin (S); whole tissues (W).
- c) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxins (TCDD); benzo(a)pyrene [B(a)P]; catalase (CAT); differentially expressed genes (DEGs); glutathione (GSH); glutathione disulfide (GSSG); glutathione peroxidase (GPx); glutathione s-transferase (GST); nanoparticles (NPs); nitric oxide (NO); superoxide dismutase (SOD); thioredoxin reductase (TrxR) toll-like receptor (TLR).

fluoranthene (32 µg/L) for 72 h [171]. Data showed that more antagonistic effects than synergic or additive effects occur in marine organisms after co-exposure to NMs and other contaminants (Table 2.1). In light of this, further studies are needed on the interactive effects of NMs with other types of NMs and other traditional and emerging contaminants, such as pharmaceuticals and PCPs in phytoplankton, zooplankton, macroalgae, marine vertebrates, and marine mammals, as well as sediment-dwelling species.

## 2.9 Environmental Risk Assessment (ERA) of NMs

In order to establish if the use of NMs is ecosafe, there is a need to establish guidelines for ERA. ERA for NMs was first based on the same criteria applied for other traditional chemical compounds [181, 182] and consists of three phases: problem formulation, planning and conducting risk assessment, and risk management. Furthermore, NMs are among the chemical compounds selected to be monitored by the European Organization for the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) of the European Union, which requires a chemical safety assessment for chemicals whose annual production is higher than 10 tonnes. The chemical safety assessment also includes four phases: registration, evaluation, authorization, and restrictions in the use [183]. However, soon it was clear that there was a need to adapt ERA to the specific nano-characteristics of NMs not included in the current ERA framework. Once the areas of uncertainty of NMs are identified, ERA can include hazard identification, dose–response assessment, exposure assessment, and risk characterization [182, 184].

ERA is function of hazard identification and dose exposure assessment. However, there are a lot of limitations and uncertainties mainly related to hazard identification and to define the most sensitive and relevant endpoints for exposure assessment particularly for inputs and environmental fate and behavior [183]. Koelmans [185] suggested that due to the uncertainties of hazard and exposure assessment, ERA of NMs should rely on prognostic approaches mainly due to the difficulty of measuring chemical concentrations and speciation of NMs. Furthermore, *in situ* exposure assessment has to rely on models to predict environmental concentrations (PECs) that are still very deficient because they do not take into account NMs dissolution by-products and their speciation in the aquatic environment [148]. Models used to estimate PECs of conventional contaminants are calibrated and validated by field and/or laboratory observations. As mentioned before, NMs may have different size, shape, and surface coatings that will affect NM behavior along with environmental characteristics (pH, NOM, dissolved oxygen, and ionic strength) [148]. However, models only predict exposure, and to determine risk, both hazard identification and exposure toxicological data are needed to consider all NMs transformations [148] and field and mesocosm data are lacking to calibrate and validate these models.

In what concerns hazard identification, a lot of gaps still exist related to the lack of scientific information and to the inadequacy of the results obtained from standard toxicity tests end points on nanotoxic effects, so it is questioned which are most suitable descriptors, and a clear interface between exposure and assessment is difficult to establish. For exposure assessment there is a debate whether at present it is possible to establish reliable dose–response relationships for NMs due to a number of factors. NMs are present in several environmental compartments (water, sediment, and biota) with a suite of forms (individuals or aggregates/agglomerates), different sizes, shape, surface area, functionalization, and coatings that can vary with time and space along with a number of biotic and abiotic factors, such as pH, salinity, exposure to sunlight, flow, and the presence of NOM [33]. Therefore, Notter et al. [186] suggested that the toxicity of metals NMs (based on  $EC_{50}$  values) is lower than the dissolved counterparts for Ag, Cu, and Zn (93.8%, 100%, and 81%, respectively). Although there were exceptions for Ag and ZnNPs, where toxicity (1.1% and 2.8%) was less than twofold higher to that of the ionic metal, it was suggested that applying a safety factor of two for these dissolved metals would protect water and sediments against the toxicity of these NMs. Although no official NMs test guidelines exist for aquatic species, similar results point out that in marine filter-feeder mollusks exposed to CuO NMs (31 nm) and QDs (6 nm) in seawater, NMs bioavailability is lower than their soluble counterparts [82, 97, 102]. Although in a more environmental realistic scenario, size and aggregation/agglomeration might be a key factor for NPs bioavailability, but biological effects are different and need to be taken into consideration in ERA. Because biomarkers seem to provide more useful information about the toxicity effects of NMs, there is an urgent need to integrate this information and use it as a relevant ecotoxicological metrics in ERA, although care should be taking in the interpretation of results. Moreover, there is a gap of information on sediments assessment of NMs; therefore, there is an urgent need to establish a relationship between the effects obtained from laboratory experiments to those occurring in the real environment.

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