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# *Daphnia magna* and mixture toxicity with nanomaterials – Current status and perspectives in data-driven risk prediction

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ABSTRACT

The aquatic ecosystem is the final destination of most industrial residues and agrochemicals resulting in organisms being exposed to a complex mixture of contaminants. Nanomaterials (NMs) are being increasingly applied in many technologies and industrial sectors, so there is an increasing concern about the negative impacts of NMs in the environment after their interaction with co-contaminants. Consequently, mixture toxicology has been gaining attention in nanotoxicology recently. Usually, mixture toxicity or combined toxicity is estimated from the individual effects of the chemicals using the mathematical models of concentration addition (CA) or independent action (IA), however these models do not account for metabolic interactions between the chemicals, when they act in related metabolic pathways and molecular targets. As NMs unique physico-chemical properties make them highly reactive with a high surface area for adsorption, those models may not realistically estimate the toxicological effects of mixtures containing NMs. The co-exposition of NMs and other environmental contaminants (e.g., organic pollutants and heavy metals) may cause different mixture effects such as addition, synergism, antagonism, or even other complicated responses, including altered toxicokinetics/toxicodynamics, which vary according to the individual components properties, environmental exposure conditions, and the biological system. Therefore, the large number of factors that may influence the toxicity of a NM and contaminant mixture makes NMs mixture risk assessment a complex task. *Daphnia magna* are one of the most commonly used model species in nanotoxicology, including in mixture studies. It's advantages include short generation time, small body sizes, ability to produce large populations rapidly, coupled with its completely mapped genome which allows the use of a multitude of omics techniques to understand the stress responses of daphnids to NMs and chemicals. Here, we analyse the toxicological effects of NMs and contaminant mixtures using *Daphnia* as a model organism, and discuss future perspectives for NMs-mixtures risk assessment focusing on harmonization of methodologies and application of data-driven science in mixture ecotoxicology.



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**Contents**



## Introduction

Mixture toxicology has recently been gaining attention in the nanotoxicology community. Due to their constant use and discard, pesticides, industrial chemicals, pharmaceuticals, and their degradation by-products are ubiquitous in the environment [1]. They co-exist as complex mixtures which have generated concern due to the lack of full knowledge and appropriate regulations about their individual and combined risks to human health and biota [2]. In addition, nanomaterials (NMs) are increasingly incorporated into new technologies and advanced materials for application in diverse fields, for example, as drug carriers, catalysts, agrochemicals, cosmetics, and electronics. Consequently, they are likely to reach the environment in multiple ways and interact with other contaminants [3,4].

The overall toxicity of chemical mixtures is related to the mode of action of each component and how they interfere with each other's metabolic pathways and molecular targets. Normally, mixture toxicity or combined toxicity is estimated as the individual effects of the chemicals using the mathematical models of concentration addition (CA), for compounds which share a mechanism of toxicity and target site(s), or independent action (IA), when the mechanisms and the targets are distinct [5]. These models assume that metabolic interactions between the chemicals do not occur, and the general effect is the combination of the individual actions. In the CA model, the toxicity is additive, that is, all components of the mixture contribute to the total toxicity depending on their concentration and potency and the mixture may present harmful effects even if all chemicals are present at levels below their individual toxicity thresholds [6]. Likewise, in the IA model the individual effects are unassociated, their combination is the sum of their biological responses, and can be calculated using the statistical concept of independent random events.

Although regulatory risk assessment frameworks for environmental chemical mixtures are based on CA or IA models [7–9], in some cases, the mixtures may present a toxicity that differs from the predicted one, and their risk to biota can be under or overestimated, if the model does not account for metabolic interactions between the chemicals [5,6]. In this context, interactions may occur at the toxicokinetic level, when one chemical interferes with the absorption, distribution, metabolism, or elimination of another compound, or at the toxicodynamic level, when the chemicals act on the same molecular or cellular processes at the same or different target sites [10]. Interactions in mixtures occur in specific combinations of components and doses and are particular to different organisms, causing higher (synergism) or lower (antagonism) toxicity than that predicted by CA or IA (Fig. 1) [11]. Detecting the possible interactions in chemical mixtures, especially synergisms, is an important and challenging task to enable proper environmental regulation, with theoretical models being a powerful tool to predict and guide experiments in that direction [12,13]. However, there is currently no model suitable for use as a standard protocol to predict mixture interactions, mainly due to the variety of detailed information regarding mode of action, metabolism (toxicokinetic and toxicodynamic data) and toxicity of chemicals and the complexity of approaches needed to obtain this data in order to parameterize such predictive data-driven models [12–14].

Beyond chemical pollutants, NMs, as emerging environmental contaminants, have been the focus of ongoing debates and research regarding their ecotoxicological impacts, and more recently, in terms of their role in enhancing the toxicity of other pollutants [3,4,15]. Due to their unique properties arising from the nanoscale, NMs physicochemically interact with organic chemicals or metals present in the environment, altering their bioavailability and resulting in mixture effects such as synergism, antagonism, addition, orother complicated responses. The different effects vary according to the properties of the individual components (i.e., NMs and chemicals), environmental conditions, and the biological system [16].

The large surface area and reactivity of NMs makes adsorption the most observed physicochemical interaction with co-pollutants. Thus, they may act as carriers influencing cell internalization, bioavailability, accumulation, and distribution of co-pollutants [4]. The responses observed are highly related to the type of interaction between the NM and the co-pollutant (e.g. complexation of metals, hydrogen bonding, electrostatic attraction, van der Waals interaction, π-π stacking, covalent bonding, etc.), the mechanism(s) of the NMs effects in the organisms and cells and the extent of desorption of the chemical co-pollutant from the NM upon contact with, or internalization by, organisms [3,16]. NMs may elicit endocytosis, disrupt cell membranes, change membrane permeability, or even interact with specific receptors facilitating pollutants entrance at higher rates than would occur for the co-pollutant alone based on equilibrium partitioning [16]. NMs may also trap chemicals through aggregation and precipitation, and induce cellular defence mechanisms that reduce the availability and/or efficacy of co-pollutants [17].

The number of characteristics (e.g., size, shape, composition, surface chemistry, reactivity, etc) of NMs that can be related to their toxicity is broad, and is further extended by modifications in the environment including biomolecular corona formation, pollutant interactions, oxidation, dissolution, agglomeration, etc., which increase significantly the complexity of their environmental risk assessment [18,19]. A recent review of the environmental dimensions of the protein corona highlighted also the evolution of the eco-corona as NMs move within the environment, are taken up and excreted by organisms and (potentially) move up the food chain,

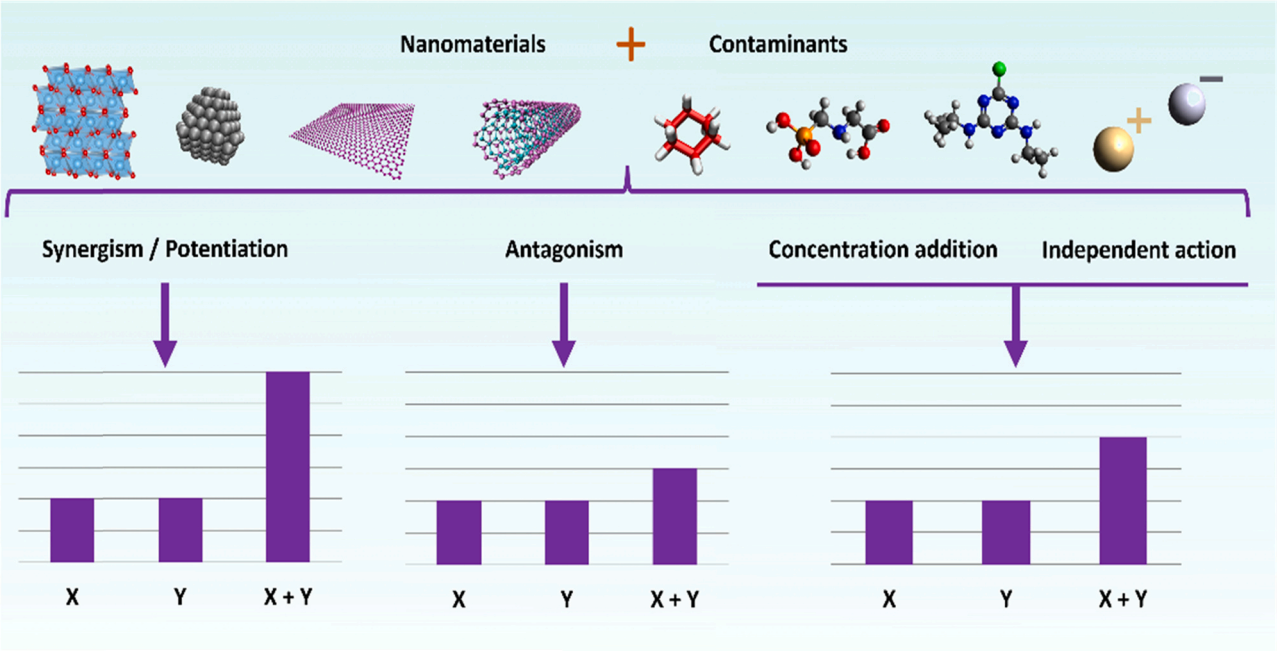


**Fig. 1.** Co-exposition of NMs with organic chemicals or metals present in the environment may result in mixture effects such as synergism, antagonism, addition, or other responses. Synergism and antagonism differentiate from concentration addition or independent action responses by the higher (synergism) or lower (antagonism) toxicity effect than expected, considering the sum of the effects of the individual components of the mixture.

exchanging biomolecules and co-pollutants along the way based on affinity and availability [20].

Ecotoxicology evaluation of NMs is based on in vivo assays utilizing a variety of test organisms, and more recently also includes some in vitro testing utilizing for example, fish cell lines [21–23]. Traditionally, in vivo studies relied on mice and rats for translation of human biology, however continued investigation into the human genome, and the sequencing of whole animal genomes, has identified significant numbers of conserved genes for growth, maintenance, and reproduction across different species. To gain insight into the environmental effects of NMs, environmentally relevant model organisms are required, and using a relevant test species can help to align eco-toxicology and human toxicology, moving towards a generalized understanding of how NMs disrupt molecular and biological processes that can be translated across species [24]. Quintessentially, a model organism will have the following traits: short generation cycles, small body sizes, a simplistic reproductive cycle with a rapid turnover of progeny to produce large populations and a simple genome [25]. 'Model organisms' are non-human species, and some of the most widely used ones in ecotoxicity and developmental biology include yeast, Drosophila, daphnids, nematodes, and zebrafish. These species are used to study molecular and biological interactions, with the intention of understanding the mechanistic significance linked to all living entities, but most importantly the associations with human biology [25,26]. Therefore, scientific investigations into these model organisms can produce meaningful data that can be generalized across multiple and higher species.

However, due to the wide variety of existing NMs and the velocity at which new materials are being developed, experimental methodologies can be expensive, labour-intensive and time-consuming, presenting limitations in relation to the volume of testing possible to be realized [27,28]. In this context, enormous efforts are being invested into the development and application of computational approaches, such as theoretical simulation and machine learning, in order to elucidate the mechanisms of NMs interactions and behaviour in biological environments, and furthermore, to assess the great volume of data, extracting information on distinct NMs properties related to complex biological responses [27,29,30] Due to the huge variability of distinct effects that NMs may induce, and the lack of full understanding of the nano-bio interface, the prediction and modelling of NM toxicity, as well as NMs joint toxicity with other chemicals can only be assessed by integrating different methodologies (i.e., experimental and computational approaches) [31].

To facilitate progress towards prediction of NMs mixture toxicity effects, this paper discusses the nanotoxicological effects of NMs and contaminant mixtures using the water flea *Daphnia magna (D. magna)* as the model organism. Additionally, promising computational methodologies with applications in environmental nanotoxicology are summarized, and their potential for extension to mixture risk assessment using data-driven science assessed.

## Daphnia ecotoxicology

Daphnia are models microcrustaceans due to their well characterized ecology, their ability to reproduce parthenogenetically (genetically identical clones) under favourable conditions [32] and to switch to sexual reproduction when under stress including from pollutants, and their fully sequenced genome [32–34]. Due to their position in the food chain, and their filter-feeding capabilities, meaning that they are exposed to everything in their environment, Daphnia are a fundamental ecological species utilized for various biological applications including acute and chronic (eco)toxicology assessment and in fundamental research on ecology, genetics, and evolution [35]. The condition of the mother has a significant influence on the phenotypic response in the subsequent offspring, via transgenerational inheritance as part of the organisms adaptation to their new environmental conditions [36]. The genetic processes that alter under chronic stress lead to phenotypic plasticity and acclimation processes [37], are easily monitored in the progeny and their subsequent generations [38], further increasing the utility of daphnids for ecotoxicity assessment.

During the last 15 years Daphnia have been utilized for nanotoxicology studies [39,40], focusing on the acute and chronic effects to life history traits when exposed to a variety of engineered (in) organic NMs. More recently, studies involving Daphnia are becoming increasingly mechanistically focused, aiming to identify NM-specific responses using a variety of transgenerational inheritance [38,41,42], multi-omics [43] approaches, and sophisticated imaging techniques. Daphnia nanotoxicology studies have also been utilized for the development and training of nanoinformatics models [44,45]. For example, Varsou et al. (2021) [44] developed a predictive ecotoxicological read-across model (a quantitative structure-activity relations (QSAR) model) for freshly-dispersed versus environmentally aged NMs, exploring the impact of NMs-surface passivation by salts and natural organic matter on the NM toxicity. Moreover, the phenotypic variation (which identifies the functional and fitness changes in the same genotype in response to NM exposure [46]) of Daphnia have been utilized by deep learning architectures to predict NM toxicity. Karatzas et al. (2020) [45] utilized light microscopy images of the daphnids acquired daily during chronic exposure to NMs over 28 days. Using non-exposed daphnids as a control, the authors were able to automatically detect malformations, such as effects of NM exposure on the length of the tail, the overall size, uncommon lipid concentrations and lipid deposits.

The use of Daphnia in multigenerational nanotoxicology studies has begun to shed important insights into adaption and/or enhanced sensitization of daphnids to NMs exposure. For example, transgenerational responses in multiple germlines exposed to different NMs have shown a direct link with maternal exposure at 'sub-lethal' effect concentrations of NMs, which were lethal to the subsequent generations [38,47]. Thus, parental exposure may compromise the sensitivity and tolerance of future generations. Indeed, long-term environmental stress disturbs physiological functions, disrupts cellular functions, and results in age related stress responses as an adaptive response to accumulated damage [48]. Use of multi-omics techniques is enabling the identification of Adverse Outcome pathways (AOPs) induced by exposure to NMs [49–51].

One proposed mechanism of toxicity of soluble inorganic NMs is via the Trojan horse effect, whereby the NMs are taken up in particulate form via receptor-mediated processes at much higher concentrations than the dissolved form would be (as this is regulated by chemical equilibrium generally) and then dissolve in vivo which disrupts the cellular molecular and biological processes [52]. A similar mechanism applies to co-pollutants, absorbed to NMs (soluble or insoluble) whereby following uptake the surface bound chemical residues/species are released, at higher concentrations than would occur via uptake of the soluble chemical, leading to toxicity. Therefore, the NMs act as a carrier to facilitate uptake, resulting in increased toxicity [4,53–55]. Other proposed mechanisms of NM mixture toxicity include facilitating bioaccumulation of NMs through co-exposure with food [56,57] leading to localized toxicity [55]. As daphnids are filter feeders it is likely that they will ingest pollutants adsorbed onto NMs via water filtration more easily than dissolved pollutants. Moreover, the specific physicochemical properties of the NMs, the biological/environmental conditions and colloidal stability will determine the type of interactions between the NMs, chemical species and the host organism to which they are exposed (Fig. 2) [58]. Additionally, the availability of biomolecules and chemicals in the environmental surroundings will also determine co-pollutant interactions with the NMs through competitive binding to the NM surface [59]. Many studies have identified the need for appropriate test media, ideally including natural organic matter or conditioned with relevant biomolecules by filtration through daphnia, to allow assessment of competitive binding and dissociation kinetics following uptake, when reflecting on realistic environmental NM exposure studies [56,60,61]. Recently, novel nano-mixture QSAR models have been used to predict the effective concentration at which 50% of organisms died ($EC_{50}$) of 76 mixtures containing $TiO_2$ NMs and one of eight inorganic/organic compounds [$AgNO_3$, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $CuSO_4$, $Na_2HAsO_4$, $NaAsO_2$, Benzylparaben and Benzophenone-3] with *D. magna* without the need for dose-response curves of the individual mixture components. The models utilized mixture descriptors ($D_{mix}$) that combine quantum descriptors of mixture components (e.g., $TiO_2$ NMs and its partners) and applied a range of machine learning techniques resulting in a random-forest model that gave better $\log EC_{50}$ prediction than either of the CA and IA models [62], indicating the enormous potential for nanoinformatics approaches in NMs mixture toxicity assessment, as discussed in detail later in this article.

## Toxicity of mixtures to *Daphnia*: effects of nanomaterials

Most nanotoxicology studies consider the effects of NMs individually and in salt-only medium. However, ecosystems are heterogeneous environments where contaminants, NMs and biomolecules will simultaneously occur. In this context, ecotoxicological studies of NMs and contaminant mixtures are a step towards a more environmentally realistic exposure scenario. The OECD classifies 4 types of chemical mixtures: intentional (e.g., specific product formulations), discharge (e.g., effluent from a specific site), coincidental (e.g., two cosmetics applied to skin) and environmental (e.g., water run-off), adding additional complexity to the challenge of assessing mixture toxicity [63]. This review will provide an overview of the toxicological effects of NM mixtures with contaminants toward *Daphnia* as the model organisms, with a focus on environmental mixtures containing NMs.

The literature research was conducted in ISI Web of Science and Elsevier Science Direct, also, cross-referenced literature from the selected studies was assessed. The terms searched were: "mixture", "joint", "combine", "co-exposure", "nano*" and "*Daphnia*". The studies found were carefully screening for eligibility (i.e. articles were only selected if the study analysed the toxicity effect of NM mixture with other substances using *Daphnia* model) and divided into three groups: carbon, inorganic and polymeric NMs.

Carbon NMs, which have unique properties, such as great thermal and chemical stability, high surface area to volume ratio and high reactivity, are promising materials for a wide range of applications, from electronics and energy to biomedicine and environmental technology. They are predominantly composed of honeycomb-structured carbon atoms [64], and due to different degrees of crystallinity and morphology, form a variety of structures, ranging from zero dimension to three dimensional nanostructures, including fullerenes, single- and multi-walled carbon nanotubes, graphite, and graphene among others (Fig. 3).

Recently, many studies have shown the potential of carbon NMs for environmental applications, such as water remediation and wastewater treatment. Due to the high adsorption capacity of those NMs, they can be applied in the removal of different types of inorganic and organic contaminants. As a result, their environmental release is inevitable. The toxicological effects of carbon NMs have been shown in many in vitro and in vivo studies, and the observed harmful effects are directly related to the physical-chemical characteristics of each type of carbon NM, such as diameter, length, shape, and surface area, among others [65]. Moreover, it has been suggested that when carbon NMs interact with contaminants by surface adsorption, the toxicity effects of both the NMs and the contaminants may be increased or decreased depending on the strength of adsorption [4].

Inorganic NMs, comprising pure metals, metal oxide, bimetallic, quantum dots and silica nanoparticles (Fig. 4), are a large group of NMs, with a wide variety of compositions, sizes, shapes, and surface chemistries [66]. Their small size and large surface area bring new properties for those NMs in comparison to their bulk counterparts,

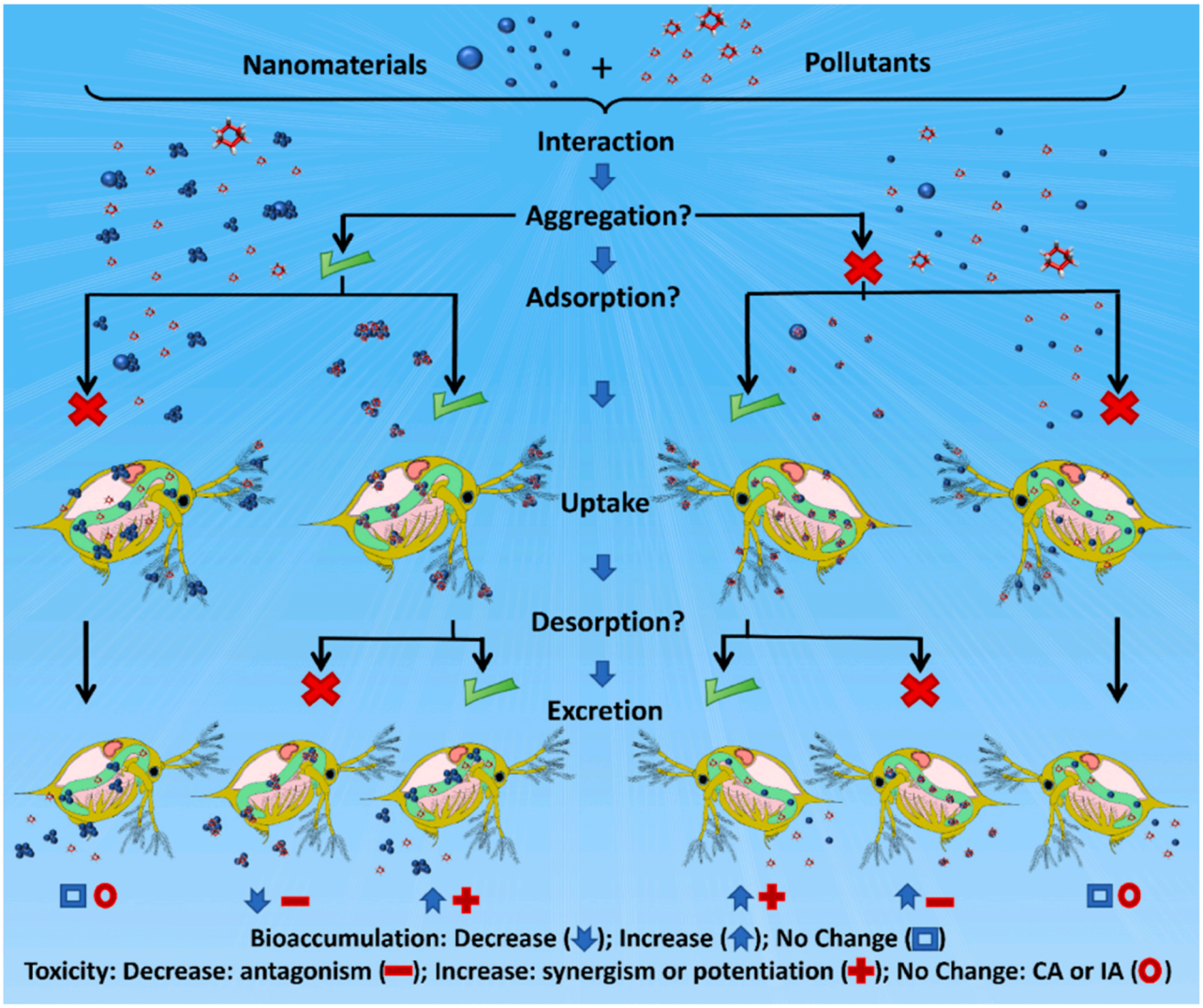


**Fig. 2.** Schematic diagram showing the different ways in which NMs co-associated with other chemical substances may influence toxicological and bioaccumulation outcomes in the model organism *Daphnia*.

enabling their applications in different fields, such as electronics, healthcare, chemical sensing, cosmetics, composites, environment, and energy [67]. Currently, according to the Nanodatabase, there are 823 commercially available products containing inorganic NMs; among these, the most used are silver, contained in 379 products, and titanium dioxide NMs, contained in 272 products [68].

Due to their great antimicrobial properties, silver nanoparticles (AgNPs) are present in several applications, ranging from disinfecting medical devices, to antimicrobial textiles, household items and in water treatment [69]. Moreover, AgNPs can be applied in biosensing and imaging applications due to their plasmon-resonance optical scattering properties [70]. Previous studies have indicated that AgNPs may cause toxicity against aquatic organisms by inducing oxidative stress and DNA damage [71]. However, once in the environment AgNPs also may interact with other contaminants causing different adverse effects. Hence, with the increased use of AgNPs-containing commercial products the concern about silver contaminants in aquatic environments is growing.

Another highly used inorganic NM is titanium dioxide ($TiO_2$). It has a high photocatalytic activity when exposed to UV radiation, and has widespread application from personal care products (sunscreens, toothpastes) and surface coatings to water treatments [72]. $TiO_2$ NMs have been used in consumer products for years, and it is estimated that in densely populated regions, the concentration of nano $TiO_2$ in the environment could reach 34–62 $\mu g\ L^{-1}$, and that the global production of nano $TiO_2$ can reach 2.5 million tons by 2025 [73,74]. Hence, the potential environmental impacts of nano $TiO_2$ have raised growing concerns. As $TiO_2$ NMs possess high reactivity and large surface area-to-volume ratios, this material has a tendency to adsorb metals and organic contaminants from natural environments. Due to its high photocatalytic activities, nano $TiO_2$ may degrade those contaminants and favour the formation of toxic by-products, influencing the toxicity of the components present in the environmental media [4,75]. Moreover, it exists in 3 allotropic forms with different structural arrangement (anatase, rutile and brookite) that directly influence its properties or toxicological response.

Polymeric NMs are another major class of NMs, constituted by a diverse range of polymers (i.e., chitosan, alginate, poly(ethylene glycol), polystyrene, and many more) [76] with a wide range of sizes, surface areas and shapes. Those materials have potential applications in theragnostic and nanomedicine applications, with promising results for targeted delivery of many therapeutics, and as imaging contrast agents [77]. In an environmental context polymeric NMs can be applied for agrochemical transport and controlled delivery [76]. While many are considered to be biocompatible, hence their biomedical applications, like every anthropogenic material

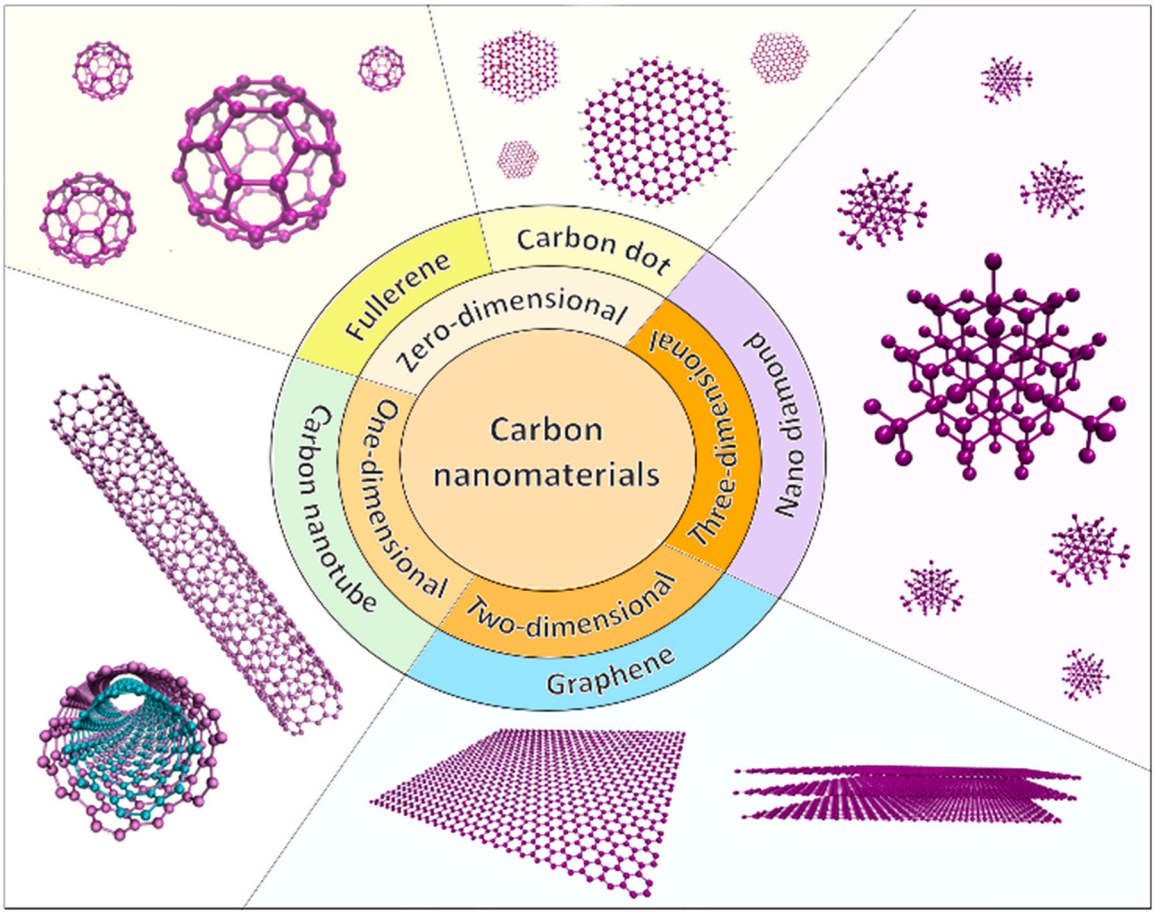


**Fig. 3.** Examples of representative carbon nanomaterial types and structures.

they can eventually reach ecosystems and interact with their components, and thus, ecotoxicological studies are essential to understand their environmental implications.

Besides carbon, inorganic and polymeric nanomaterials we also have combination of more than one nanomaterial forming nanoheterostructures (NHs), which comprise two or more components fused into one nanoparticle [78–83]. Those structures are gaining attention, as the combination of different nanoparticles with multiple properties allows the synthesis of single nanoscale structures with multiple and increased functionalities; the combination of properties not only sum the properties of the components but also creates new properties. Therefore, those materials are being applied in several areas, such as catalysis, electronics, optoelectronics, medicine, biology, etc. There are a few toxicology studies on the effects of nano-heterostructures towards daphnia [84–88]. However, none focus on co-exposure, therefore this remains a gap that still needs to be further addressed.

Understanding how NMs interact with other environmental pollutants and their combined impacts on organisms is an emergent research and regulatory question. Nevertheless, this type of study is not trivial as the potential of NMs to interact with different types of contaminants strongly depends on several factors, such as the NM's physical/chemical properties, its colloidal stability, the environmental conditions (i.e., pH, temperature, ionic strength and available biomolecules), as well as the structures and properties of the contaminants (Fig. 5) [4]. Despite the fact that the importance of the biomolecule corona in mediating NMs toxicity is well accepted in medical and nanosafety research, environmental acceptance and adjustment of NMs ecotoxicity studies lags behind [89,90], and the role of the eco-corona in NMs mixture toxicity studies is almost completely ignored to date [91]. Thus, few studies address those factors sufficiently, with most of the literature to date assessing binary mixtures in salt-only media and their toxicological outcomes.

### *Carbon nanomaterials*

The potential of carbon NMs to adsorb organic and inorganic pollutants is well known; for example, they have a high capacity to absorb heavy metals such as Zn, Cr, Pb, As, Hg [92,93], organic pesticides such as phenols [92,94,95], diuron and dichlobenil [96,97], atrazine [98,99], and even antibiotics such as tetracycline [100], and ciprofloxacin [101]. However, the adsorption of pollutants can be influenced by different physical-chemical properties, such as media composition, pH, NMs properties, and chemicals properties [4,102], and in natural environments, there will also be competitive interactions with natural organic matter (NOM) and other biomolecules [59,103]. For instance, surface functionalization has a major contribution on the interaction of carbon NMs and pollutants. Acid treatment introduces defects into C=C bonds and adds different functional groups to the carbon nanotube (CNT) structure, increasing its hydrophilicity and enhancing their adsorption potential [104]. Chemical oxidation of graphene also enhanced the adsorptive characteristics by adding oxygen-containing functional groups (carboxyl, hydroxyl and epoxy groups) [105]. Additionally, the structural characteristics of the carbon NM can also impact on their adsorption capability, for example, CNT can adsorb pollutants on their external and internal surfaces, therefore the amount of adsorption sites can be enhanced by adding walls to CNTs, such that multiwalled MWCNT have additional adsorption spaces between their walls. In the case of graphene oxide (GO), adsorption can be enhanced by adding layers

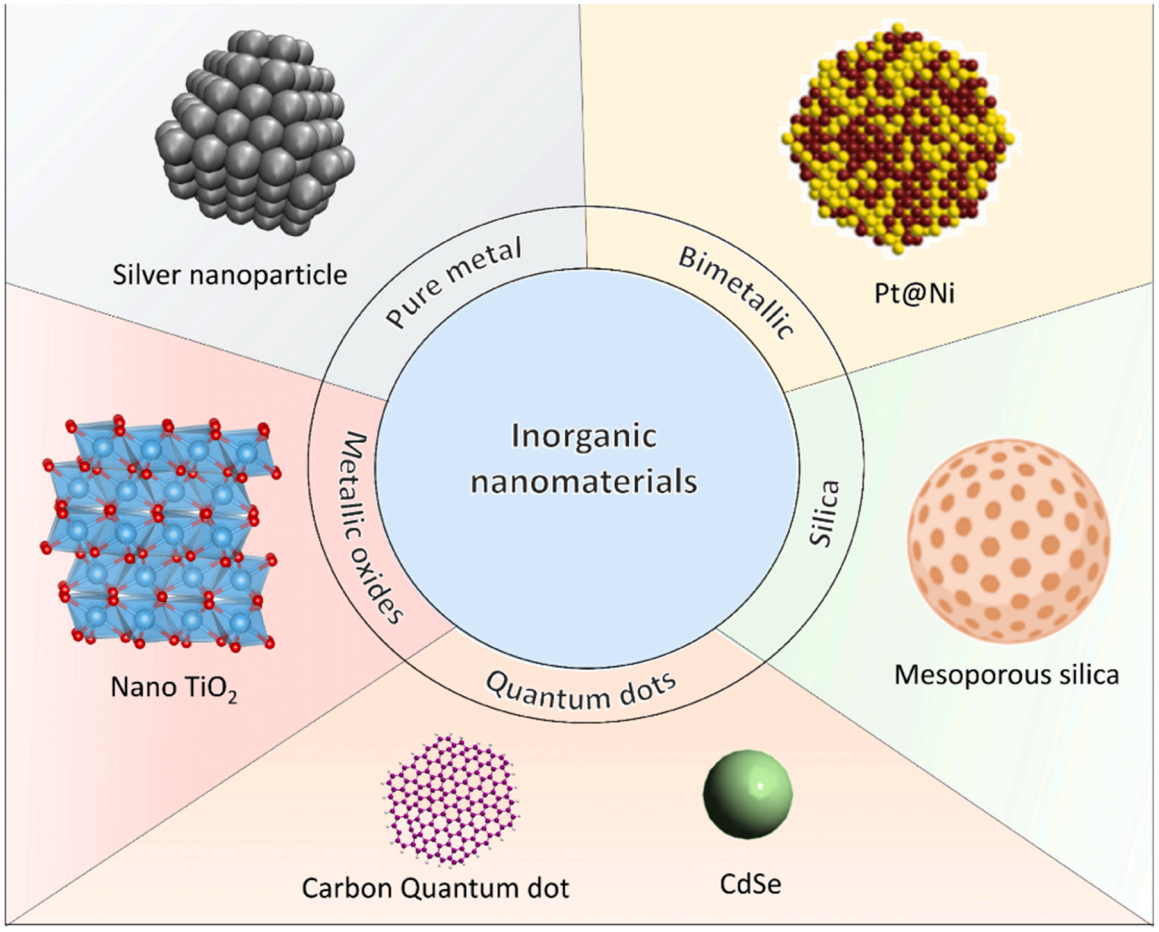


**Fig. 4.** Examples of the classes of inorganic nanomaterials and individual examples.

to its structure as the adsorption can occur between layers as well as on the external surface [106].

Due to those properties carbon NMs have an important role in the partitioning, transport, and toxicity of other contaminants [107]. The interaction between carbon NMs and aquatic pollutants can enhance or suppress the toxicity of those chemicals depending on how strongly the co-pollutants are bound to the NM surface and on the persistence and bioaccumulation or clearance of the NMs. The study of these effects is environmentally relevant and extremely necessary to predict the impact of carbon-based NMs on the aquatic environment over acute and chronic timescales. In this review, we find only 18 studies that explore the co-exposition of carbon NMs and pollutants towards the model species daphnia. Among those studies, 5 address fullerene, 9 CNTs, 3 graphene and 1 graphite-diamond (Table 1).

Different effects can be observed in the studies that analysed the influence of fullerene on the toxicity of contaminants. Baun et al. studied the toxicological effect in *D. magna* of an aged dispersion of fullerene and its co-exposition with three organic pollutants: Methylparathion, Phenantherene and Pentachlorophenol (PCP). In this case, fullerene decreased the toxicity of phenantherene and PCP and did not significantly influence the toxicity of methylparathion [108]. In the assays, fullerene was present in the form of aggregates (> 200 nm) and showed high adsorption of phenantherene (85%) and PCP (10%), therefore the observed effect on the toxicity of these chemicals could be related to a decrease in their bioavailability in the NM's presence. On the other hand, Brausch et al., studying the influence of C60 on two organic pollutants toxicity, Bifenthrin and Tribufus, observed that the NM increased the toxicity of both pollutants in the *D. magna* immobilization assay [109]. Both studies used Sigma-Aldrich $C_{60}$ however in the Brausch studies the $C_{60}$ were functionalized. It is known that functionalization often decreases the hydrophobicity of the materials, consequently increasing their dispersion; some functional groups also serve as binding sites for organic pollutants and for metal ions. Thus, binding to NMs may increase the bioavailability and uptake, causing a trojan horse effect. This was also observed by Tao et al., where stable aqueous fullerene nanocrystals increased the toxicity of $Cu^{2+}$ and significantly enhanced the activity of $Cu^{2+}$-ATPhase, an enzyme indicative for copper transportation in organisms, therefore increasing the bioavailability and uptake of $Cu^{2+}$[110].

Heidari et al. studied the toxicity of $C_{60}$ in the solvent 1,2,4-trimethylbenzene (TMB) versus olive oil, sunflower oil and linseed oil. The toxicity effect observed was directly related to the $C_{60}$ concentration, that is, at lower concentrations of $C_{60}$ the mortality was reduced, while for higher concentration it was increased (except for sunflower oil) [111]. The presence of $C_{60}$ inside and outside *D. magna* was evaluated; in combination with TMB it was observed that fullerenes aggregated in *D. magna's* gut, while for olive, sunflower and linseed oil, the $C_{60}$ was observed inside and outside the gut, varying according to the concentration of $C_{60}$, therefore the uptake may be influenced by the properties of the organic compounds (solvent) as well as the NM properties (Fig. 6). $C_{60}$ has a large adsorption capacity, especially for hydrophobic compounds as the strong ionic forces in solution favour the interaction with non-polar compounds. Also, $C_{60}$ has the capacity to translocate through the daphnia gut and even through the plasma membrane acting as a carrier for those types of pollutants, causing a trojan horse effect. This was observed by Seke

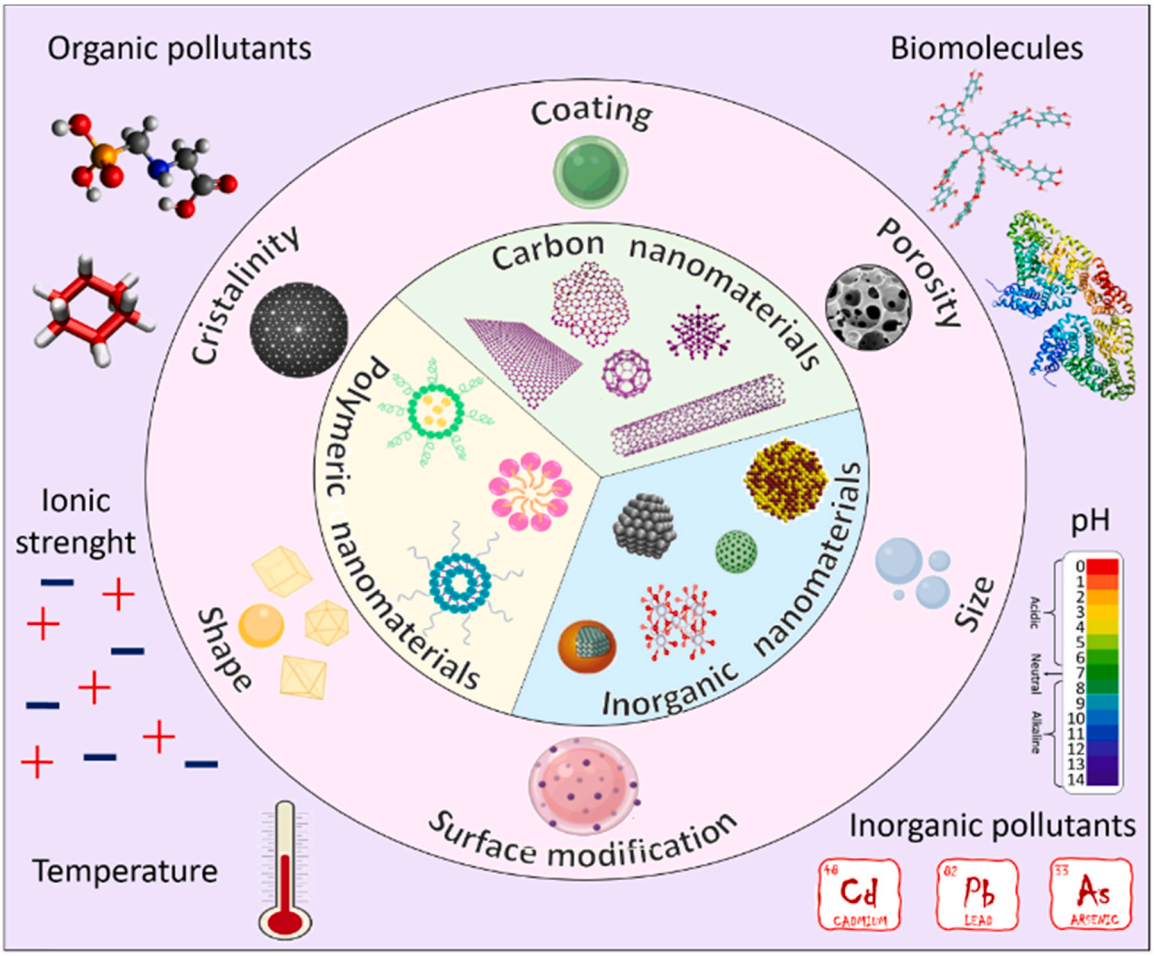


**Fig. 5.** Environmental conditions and physical-chemical properties that influence the toxicological profile of NMs and their environmental mixtures.

et al. studying the combined toxicity of $C_{60}$ with chloromethanes. $C_{60}$ caused a synergistic mitotoxicity in *D. magna* midgut epithelial cells. *Daphnia* are filter-feeders, therefore NMs in water may be ingested causing damage to its digestive tract. In this sense, it was observed that $C_{60}$ does not cause toxicity to *D. magna* gut, however, when combined with chloromethanes the midgut epithelium damage caused by chloromethanes was more pronounced, causing necrosis, mitochondrial swelling and cristolysis. Thus, $C_{60}$ may cause a trojan horse effect by facilitating the internalization of chloromethanes in *D. magna* digestive tract epithelial cells thereby increasing their toxicity [112].

The majority of studies about co-exposure of carbon NMs and pollutants address the influence of CNTs on the toxicological effects of chemicals towards daphnids. There are two types of CNTs, single walled (SWCNT), that consist of a wrapped monolayer of graphene, and multi walled (MWCNT), that consist of several single walled tubes wrapped one over another [126]. Within those classifications, CNTs can present different physical and chemical properties, such as diameter, length, flexibility, number of layers, and can be functionalized with different surface groups. Thus, the toxicity effects may differ depending on the properties of the CNT. This was observed by Yu et al. studying the influence of CNTs (single and multiwalled) with and without -COOH functionalization on the bioaccumulation of $Cd^{2+}$ and $Zn^{2+}$. In this study, the functionalized MWCNT and SWCNT increased the bioaccumulation of $Cd^{2+}$ and $Zn^{2+}$ while without functionalization both CNT decreased bioaccumulation [115]. They also studied the adsorption capacity of each CNT, and for the non-functionalized SWCNT and MWCNT there was almost no adsorption of $Cd^{2+}$ and $Zn^{2+}$, while for the functionalized SWCNT and MWCNT (4 mg $L^{-1}$) 50% and 20% adsorption of $Zn^{2+}$ and 60% and 20% of $Cd^{2+}$, respectively, were determined. Therefore, the increase in toxicity caused by the functionalized CNT could be characterized as a trojan horse effect, in which the functionalized CNT carrying $Zn^{2+}$ and $Cd^{2+}$ are ingested by daphnia, facilitating enhanced uptake of the metals.

Moreover, Liu et al. studied the bioaccumulation and toxicity of $Cd^{2+}$ when exposed with 2 types of -COOH functionalized MWCNT, one long (10–20 µm) and one short (0.5–2.0 µm) [118]. In both cases, compared with cadmium exposure only, a decrease in $Cd^{2+}$ bioaccumulation was observed, also, the long MWCNT decreased the acute toxicity of $Cd^{2+}$ to *D. magna*, while the short MWCNT did not significantly affect toxicity. In this study the adsorption of $Cd^{2+}$ was < 11% for short-MWCNT and < 17% for long-MWCNT. At the same concentration the long MWCNT solutions were much darker than the short MWCNT solutions. The authors suggested that the decrease in $Cd^{2+}$ bioaccumulation and toxicity may be associated with inhibition of the uptake of $Cd^{2+}$ because of the free metal ions binding with CNT decreasing bioavailability. However, due to the low sorption potential of MWCNT in this study, they also suggested that in the dark environment, caused by the presence of the MWCNT in medium, the organisms were less active, lowering their mobility, metabolism and filtering rate, influencing the uptake, explaining the different results for both MWCNT.

Besides NM physical and chemical properties, several environmental factors may influence the adsorption capacity of NMs in the environment. For example, Lee et al. studied the influence of two functionalized MWCNT (-COOH and $-NH_2$) on $Cd^{2+}$ toxicity and showed that both MWCNT decreased the toxicity of the metal [121].

**Table 1**
Papers addressing the toxicity of carbon NMs and pollutants towards *Daphnia* model.

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Paper |
|---|---|---|---|---|---|---|---|---|
| Fullerene (C60) | Purchased from Sigma-Aldrich.98% purity.Not further characterized. | Methyl parathion, pentachlorophenol (PCP) and Phenanthrene | Elendt M7 medium | C60: 5 and 8 mg $L^{-1}$PCP: 0.1 – 100 µg $L^{-1}$Phenanthrene: 10 – 10,000 µg $L^{-1}$Methyl parathion – data not showed. | Acute immobilization(48 h) Bioaccumulation | *D. magna* < 24 h (neonate) | C60 reduced the toxicity of PCP and phenanthrene and did not alter the toxicity of methyl parathion | Baun et al. [108] |
| Functionalized fullerene (fC60) | Purchased from Sigma-Aldrich.Not further characterized. | Bifenthrin and tribufos | US EPA 2001 | fC60: 52.75 µg $L^{-1}$Bifebthrin: 0.24 – 2.25 µg $L^{-1}$Tribufos: 1.59 – 25.40 µg $L^{-1}$ | Acute immobilization (48 h)Growth inhibition(10 days) and Reproduction(70 and 21 days) | *D. magna* < 24 h (neonate) | fC60 increased the acute toxicity of bifenthrin and decreased for tribufos. However, there were no significant differences for the chronic endpoints in both cases. | Brausch et al. [109] |
| Fullerene nanocrystal (nC60) | Purchased from Material Electronic Research corporation.Not further characterized. | $Cu^{2+}$ solution | US EPA 2004 | nC60: 100 µg $L^{-1}$$Cu^{2+}$: 1 – 42 µg $L^{-1}$ | Acute immobilization(48 h) Bioaccumulation | *D. magna*5 days old | C60 enhanced Cu toxicityC60 facilitated accumulation. | Tao et al. [110] |
| Fullerene (nC60) nanoaggregates | Purchased from Sigma-Aldrich, 99.5% purity. Irregular shape. nC60 diameter: 20–30 nm. nC60 aggregates size distribution: 210–1520 nm (after the material were aged for 50 days in artificial fresh water in the dark at 22 °C) | Chloroform (TCM) and dichloromethane (DCM) | SOP 2007 | nC60: 40 ± 2 mg $L^{-1}$TCM:105.1 and 200.8 mg $L^{-1}$DCM:103.5 and 206 mg $L^{-1}$ | Toxicity in midgut epithelial cells | *D. magna (adults)* | C60 increase the effect of TCM and DCM on D. magna mid gut epithelial cells. | Seke et al. [112] |
| Fullerene (C60) | Purchased from MER (Tuscan, AZ).99% purity. | 1,2,4-trimethylbenzene (TMB), linseed oil,olive oil, and sunflower oil | US EPA | C60: 176, 88, 44, 22, and 11 mg $L^{-1}$ mg $L^{-1}$TMB: 707.8 mg $L^{-1}$Linseed oil: 17,358.2 mg $L^{-1}$Olive oil: 54,485.4 mg $L^{-1}$Sunflower oil: 159,920.7 mg $L^{-1}$ | Acute immobilization (48 h) | *D. magna* < 24 h (neonate) | Linseed oil toxicity increase with increasing C60 concentrations. For TMB, olive and sunflower oil, the toxicity was lowered with low concentrations of C60. | Heidari et al. [111] |
| Single-walled carbon nanotubes with surface modification (SWNTs) | Metallic residue: 20%; Diameter: 1.53 nm; Hydrodynamic size: 582.6–1614 nm;Zeta potential:−1.6 × $10^{-9}$ to −1.4 × $10^{-9}$ $m^2V^{-1}s^{-1}$ | $Cu^{2+}$ (does not say if it is salt or solution) | US EPA | SWNTs: 0.5 – 10 mg $L^{-1}$$Cd^{2+}$: 25 – 525 µg $L^{-1}$ | Acute toxicity(48 and 96 h) Bioaccumulation | *D. magna* < 24 h (neonate) | SWCNT with Lysophosphatidylcholine (LPC) surface modification enhanced the toxicity of Cu. | Kim et al. [113] |
| Single walled carbon nanotubes (SWCNT) | Diameter: 1.53 nm; hydrodynamic size:582.6–1614 nm; Electrophoretic mobility: −1.6 × $10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ to −1.4 × $10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ (mean = −1.1 ± 0.1 SD) | $CdCl_2$ ($Cd^{2+}$) | ASTM | SWCNT: 0.5–10 mg $L^{-1}$$Cd^{2+}$: 25–525 µg $L^{-1}$ | Acute toxicity(48 h) | *D. magna* < 24 h (neonate) | SWCNT enhanced the toxicity of Cd. | Revel et al. [114] |

(*continued on next page*)

**Table 1** (*continued*)

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Paper |
|---|---|---|---|---|---|---|---|---|
| Multi and single-walled Carbon nanotubes with (F-MWCNT and F-SWCNT) and without (NF-MWCNT and NF-SWCNT) oxygen groups and defects | F-SWNTs and NF-SWCNT: 90% and 95% purity, respectively, 0.8–1.6 nm ID; 1–2 nm OD; length: 5–30 µm for both; 2.73% of Carboxyl groups for F-SWNTsF-MWNTs and NF-MWNTs: 90% and 95% purity, respectively; 8–10 nm ID; 10–20 µm of length for both and 3.86% of carboxyl groups for F-MWNTs | $CdCl_2$ ($Cd^{2+}$)$ZnCl_2$ ($Zn^{2+}$) | Modified SM7 (low $Ca^{2+}$ concentration) medium | NF-SWNTs and NF-MWNTs: 0.5 – 4 mg $L^{-1}$F-SWNTs and F- MWNTs: 1 – 4 mg $L^{-1}Cd^{2+}$: 0.5 µg $L^{-1}Zn^{2+}$: 5 µg $L^{-1}$ | Bioaccumulation | *D. magna*7 days old | CNT decreased metal uptake while functionalized-CNT increase metal uptake. | Yu and Wang [115] |
| Hydroxylated multiwalled carbon nanotubes (OH-MWNTs) | OD: 20–40 nm; ID: 5–10 nm; Length: < 5 µm; Purity: > 97%; Ash: < 3%; specific surface area: 90–120 $m^2$ $g^{-1}$; OH content: 1.8% | $Ni(NO_3)_2.6H_2O$ ($Ni^{2+}$) | Chinese standard (GB/T 1326 6–1991) medium | OH-MWNTs: 0.1 – 5 mg $L^{-1}Ni^{2+}$: 1 – 8.1 mg $L^{-1}$ | Acute immobilization(48 h) Bioaccumulation | *D. magna* < 24 h (neonate) | OH-MWCNT increased toxicity and bioaccumulation of Ni. | Wang et al. [116] |
| Hydroxylated multi-walled carbon nanotubes (OH-MWNT) | OD: 36 ± 2 nm; length 4 ± 1.5 µm; purity: > 97; Ash: 2 ± 3%; 0 point charge: 6.1; Specific surface area: 115 ± 5; Hydroxyl group content: 1.82% | $Na_3AsO_4.12H_2O$ (AS (V))$As_2O_3$ (AS(III)) | OECD guideline | OH-MWNT: 0.1 – 5 mg $L^{-1}$AS (V) and AS(III): 100 – 400 µg $L^{-1}$ | Acute immobilization(48 h) Bioaccumulation | *D. magna* < 24 h (neonate) | OH-MWCNT enhance arsenic toxicity and bioaccumulation. | Wang et al. [117] |
| Functionalized multi-walled carbon nanotubes (F-CNT) and Short Functionalized multi-walled carbon nanotubes (SF-CNT) | -COOH: 2%; Purity: > 95%; ID: 5–10 nm; OD: 10–20 nm; specific surface area: > 200 $m^2$ $g^{-1}$; electric conductivity: > 100 s $cm^{-1}$; length: F-CNT: 10–30 µm and SF-CNT: 0.5–2 µm | $CdCl_2$ ($Cd^{2+}$) | Simplified Elendt M7 medium(low $Ca^{2+}$ concentration) | F and SF-CNT: 4 and 8 mg $L^{-1}Cd^{2+}$: 2–20 µg $L^{-1}$ | Acute toxicity(72 h)Uptake assay(8 h) | *D. magna*7 days old | Short F-MWCNT did not affect Cd toxicity, while long F-MWCNT decrease Cd toxicity.Both F-MWCNT reduced Cd accumulation. | Liu and Wang [118] |
| Multiwalled carbon nanotubes (MWCNT) | Obtained from Bayer Material Science;Purity: > 95%; mean diameter: < 13 nm; Inner diameter: from 4 nm to several µm | 3,4,4'-trichlorocarbanilide (TCC) | ElendtM4 medium | MWCNT: 1 mg $L^{-1}$TCC: 25 – 61 µg $L^{-1}$ | Acute immobilization(48 h) | *D. magna* < 24 h (neonate) | The toxicity of TCC was reduced in the presence of MWCNT. | Simon et al. [119] |
| Carboxylate and polyethyleneimine modified Multi-walled carbon nanotubes (C-MWCNT and N-MWCNT, respectively) | Purchase from NanoLab Inc.; surface area: C-MWCNT 194 $m^2$ $g^{-1}$ and N-MWCNT 118.8 $m^2$ $g^{-1}$; Primary size: 15 ± 5 nm diameter and 1–5 µm of length for both MWCNT; hydrodynamic diameter: C-MWCNT: 186.2 ± 6.8 and N-MWCNT 443.5 ± 16.1; zeta potential: C-MWCNT: −40.3 ± 0.4 and N-MWCNT 25.2 ± 0.6 | $Pb^{2+}$ (does not say if it is salt or solution) | Modified 10-time diluted M4 medium with no EDTA | MWCNT: 20 mg $L^{-1}Pb^{2+}$: 0.01 – 2.4 mg $L^{-1}$ | Acute immobilization(48 h) Bioaccumulation | *D. magna* < 24 h (neonate) | Negatively charged C-MWCNT significantly decreased Pb toxicity.Positively charged N-MWCNT did not significatively change Pb toxicity. | Jang and Hwang [120] |

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**Table 1** (*continued*)

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Paper |
|---|---|---|---|---|---|---|---|---|
| Carboxylic and amino Multi-walled carbon nanotubes (COOH_MWCNT and $NH_2$-MWCNT, respectively) | Purchased from NanoLab Inc; Purity: > 95%; OD: 15 ± 5 nm; length: COOH_MWCNT: 5 – 10 nm and $NH_2$-MWCNT: 5 – 20 nm; Zeta potential: COOH-MWCNT −62.1 ± 0.7 and $NH_2$-MWCNT −32.2 ± 0.4 | $CdCl_2$ ($Cd^{2+}$)Natural kaolinite | US EPA guidelines | COOH_MWCNT and $NH_2$-MWCNT: 5 mg $L^{-1}$$Cd^{2+}$: 10 – 500 µg $L^{-1}$ | Acute immobilization(48 h) | *D. magna* < 24 h (neonate) | In a binary system, MWCNT and Kaolinite decrease Cd toxicity. However, in a ternary system the toxicity was increase. Consequently, kaolinite negatively affect the adsorption of Cd on MWCNT. | Lee et al. [121] |
| Graphene oxide (GO) | > 99.5% purity; 1 – 1.77 nm thickness; 1 – 5 layers; surface area: 300 – 450 $m^2g^{-1}$ | $CuCl_2$·2 $H_2O$ ($Cu^{2+}$)$CdCl_2$.2.5 $H_2O$ ($Cd^{2+}$)$ZnCl_2$ ($Zn^{2+}$) | US EPA standard methods | GO: 2 mg $L^{-1}$$Cu^{2+}$: 5 – 80 µg $L^{-1}$$Cd^{2+}$: 10 – 100 µg $L^{-1}$$Zn^{2+}$: 250 – 1500 µg $L^{-1}$ | Acute toxicity(72 h) BioaccumulationOxidative stress (SOD, GSH and MDA) | *D. magna*5 days old | GO mitigated the toxicity, decreasing metal bioaccumulation. | Ni and Li[122] |
| Graphene (GN) and graphene oxide (GO) | GN: 98% purity; 0.8 – 1.2 nm thickness; 0.5 – 5 µm of lateral sizeGO: 99% purity; 0.8 – 1.2 nm thickness; 0.5 – 5 µm of lateral size | $Cu(NO_3)_2$ ($Cu^{2+}$) | ElendtM7 medium | GO: 2 mg $L^{-1}$$Cu^{2+}$: 10 – 200 µg $L^{-1}$ | Acute immobilization(48 h) Oxidative stress (MT, SOD, GSH-PX and MDA) | *D. magna* < 24 h (neonate) | GN increased Cu accumulation, however GO decreased Cu toxicity and bioaccumulation. | Liu et al. [123] |
| Graphene oxide (GO) | Purchase from Aladdin Industrial Co. (Shanghai, China). Thickness:0.55 – 1.20 nm; platelet size: 0.5 – 3.0 µm | $ZnCl_2$ ($Zn^{2+}$)Zn NP (size: 14 nm; surface area: 30 ± 5 $m^2$ $g^{-1}$; purity > 99%) | OECD 202 guidelines | GO: 1 – 80 mg $L^{-1}$$Zn^{2+}$: 0.125 – 8 mg $L^{-1}$Zn NP: 0.01 – 0.4 mg $L^{-1}$ | Acute immobilization(48 h) Oxidative stressROS | *D. magna* < 24 h (neonate) | The joint effects of ZnO NPs and GO NPs were additive to *D. magna.* | Ye et al. [124] |
| Graphene oxide (GO) and BSA coated graphene oxide (BSA@GO) | GO: < 1.5 nm thickness; 0.24 ± 0.01 nm surface roughness; 18 – 308 nm flake size; ~32% oxygen contentBSA@GO: ± 3 nm thickness; 1.22 ± 0.26 nm surface roughness | $CdCl_2$ ($Cd^{2+}$) | ABNT NBR 12713:2016 guideline | GO and BSA@GO: 1 – 100 mg $L^{-1}$$Cd^{2+}$: 0.1 – 2.1 mg$L^{-1}$ | Acute immobilization (72 h) | *D. magna* < 24 h (neonate) | GO decrease the toxicity of Cd2 +, and BSA coated GO enhanced the mitigations of $Cd^{2+}$ acute toxicity. | Martinez et al. [91] |
| Graphite-diamond NP (GDN) | Purchased from PlasmaChem (Germany); Diamond content: > 20%; Ash content: < 3%; Average particle size: 4 nm; free of metals and organic impurities | Thiabendazole (TBZ) | OECD 202 guidelines | GDN: 1.56 – 50 mg $L^{-1}$TBZ: 0.16 – 5 mg $L^{-1}$ | Acute immobilization(48 h) | *D. magna* < 24 h (neonate) | At low doses GDN enhanced the toxicity of TBZ, while at high doses GDN decreased the toxicity of TBZ. | Martín-de-Lucía et al. [125] |

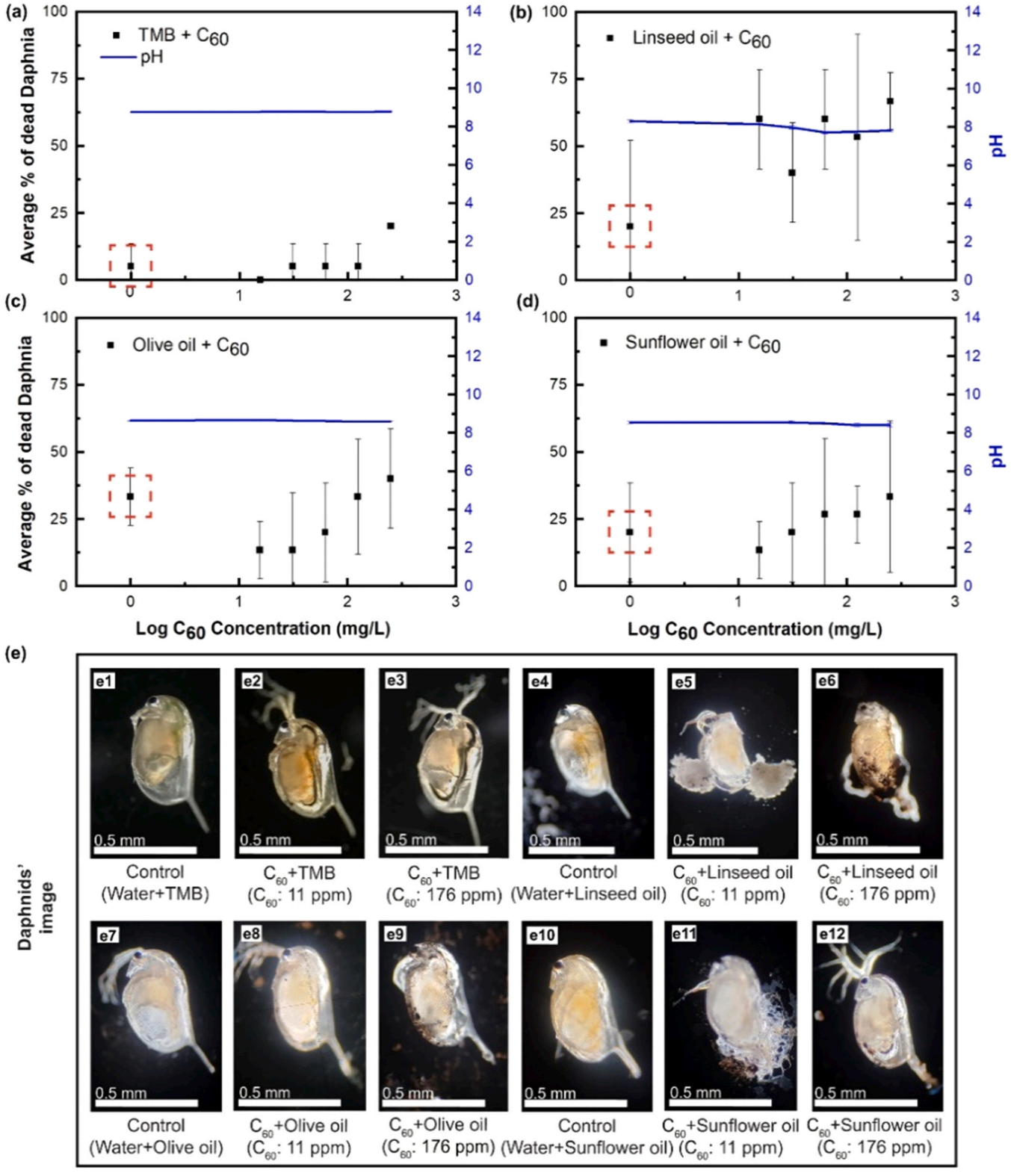


**Fig. 6.** The pH of the test medium at the end of each experiment and the average mortality of daphnids for the acute combined toxicity experiments. Daphnids were exposed to (a) TMB+nC60, (b) linseed oil+ nC60, (c) olive oil+ nC60, and (d) sunflower oil+ nC60. The average mortality of daphnids exposed to the control (50% solvent and 50% reconstituted water) for each mixture is shown in the red box. The average percentage of dead daphnids with 95% confidence intervals was used for (a)–(d). (e) The morphology of daphnids after 48 h of exposure to combined toxicants (C60+ solvents) and controls (reconstituted water+solvents). In the figure caption, water=reconstituted water (reproduced from ref. [96] with permission provided from Elsevier and Copyright Clearance Center 2021).

However, by adding kaolinite, a clay mineral present in natural waters, to the mixture a decrease in $Cd^{2+}$ adsorption capacity by both MWCNT and a consequent increase in daphnid mortality was observed. The authors suggested that kaolinite reduces MWCNT mobility and triggers aggregation reducing the adsorption of $Cd^{2+}$. This shows the importance of the environmental conditions for a more accurate prediction of the impact of mixtures in the aquatic ecosystem.

The pH is another important parameter to be taken into consideration when studying adsorption, and consequently, plays an important role in the toxicity of mixtures. This was shown by Wang et al., studying the influence of pH on the toxicity of a mixture between -OH functionalized MWCNT and $Ni^{2+}$ [116]. Overall, OH-MWCNT increased the toxicity and bioaccumulation of $Ni^{2+}$, however this effect was more pronounced at lower pH. Similarly, Wang et al. studied the influence of pH on a mixture of -OH functionalized MWCNT and As (III) and As (V) [117]. The adsorption capacity for As (III) was low and did not significantly change with pH, however, an increase in toxicity when increasing pH values (6, 7 and 8) was observed. On the other hand, OH-MWCNT had a larger adsorption capacity for As (V), and pH greatly influenced this: as pH increased the adsorption capacity for this metal decreased, and consequently there was less uptake and thus less toxicity, which also decreased as the pH increased. These results demonstrated that adsorption of the co-pollutant to the NM is a key factor in mixture toxicology.

In the case of graphene there are only three studies approaching the mixture toxicity in *D. magna*. Ni and Li studied the toxicity of graphene oxide (GO) with $Cu^{2+}$, $Cd^{2+}$ and $Zn^{2+}$ at different pHs (7.8 and 6.8) [122]. Overall, their results show that GO decreased toxicity and reduced metal bioaccumulation by daphnids. In this study, pH played an important role in toxicity. In all treatments the toxicity increased when pH decreased from 7.8 to 6.8. The low pH decreased metal adsorption by GO and increased the desorption therefore increasing toxicity and bioaccumulation by *D. magna*. The study also reported oxidative damage using three biochemical indicators: superoxide dismutase, malondialdehyde and glutathione. For all three a reduction in oxidative damage when metals were co-exposed with GO was observed, confirming the toxicity data.

Functional groups also have a high impact on graphene toxicity when combined with pollutants. Liu at al. studied the role of surface oxygen functional groups on the toxicity effect of graphene (GN) and graphene oxide (GO) mixtures with $Cu^{2+}$ towards *D. magna* [123]. Oxygen functional groups increased the adsorption capacity and water stability, therefore GO reduced *D. magna* mortality, $Cu^{2+}$ bioaccumulation and oxidative stress, while GN showed enhanced oxidative stress, increased bioaccumulation of $Cu^{2+}$ and did not significantly influence $Cu^{2+}$ toxicity. The adsorption capacity of GO can also be increased by the formation of a protein corona, as shown by Martinez et al., where it was observed that BSA corona coated GO (BSA@GO) increased the $Cd^{2+}$ adsorption capacity 4.5 fold compared with GO. Consequently, BSA@GO enhanced the mitigation effect of $Cd^{2+}$ toxicity when compared with GO [91].

Due to the extensive use of NMs, mixtures of NMs of different compositions may occur in natural ecosystems. Ye at al. studied the toxicological effect of a mixture of Zn nanoparticles (Zn NP) and GO, considering the contribution of NMs and dissolved ions release to the overall toxicity. Their findings suggest an additive response in *D. magna* [124]. The results implied that suspended particles have a higher influence on the combined toxicity than released $Zn^{2+}$, mainly because of the ions released from the Zn NPs being adsorbed onto the GO, which, in the medium used for the *D. magna* immobility assay, have a sorption capacity of 25% and 17% when the GO concentrations were 1 and 100 mg $L^{-1}$, respectively.

Martín-de-Lucía et al. studied the combined toxicity of graphite-diamond (GDN) NPs and thiabendazole (TBZ) to *D. magna* in the presence and absence of food [125]. Their results show that at low concentrations GDN increased the toxicity of TBZ, while at higher GDN concentrations a decrease in toxicity was observed. The authors attributed this effect to the GDN agglomeration at higher concentrations leading to lower uptake by daphnids, whereas at lower GDN concentrations the particles were more bioavailable, therefore causing a synergic effect. The presence of food in the assay reduced toxicity and the synergic effect, probably due to the adsorption of GDN and TBZ onto aggregates of food. Therefore, food is also an important factor to consider when studying NMs and co-pollutant mixtures. The presence of food helps with NM elimination from the digestive tract [90], thus the presence or absence of food can cause an under or overestimation of NM bioaccumulation and toxicity.

Based on the studies so far, it is not possible to draw conclusions as to whether carbon NMs increase or decrease the toxicity of aquatic pollutants towards daphnia. This is not only because of the wide range of properties that NM and co-pollutnats can have, but also due to the lack of standardization in the toxicological assessment of NMs where test guidelines are still evolving, and in the characterization of NMs studied. This makes it difficult to compare the obtained results. For example, there are different guidelines for toxicological assays in different geographical and regulatory regions; while the fundamentals of those guidelines are the same, each of them has minor differences that may impact the toxicological assessment, especially for NMs whose properties are so strongly influenced by their surroundings, which has been described as NMs having extrinsic properties [127,128]. For instance, it is known that the medium used for the assay strongly influence the NM colloidal stability, and this directly impacts the toxicological outcomes [129]. In the studies addressed in this review, the authors used different guidelines (USEPA, OECD, ASTM, Chinese Standard) and different media, as shown in Tables 1–3. Besides, in some studies, the ion composition of the medium was changed to improved NM colloidal stability. For example, Yu and Wang [115] and Liu and Wang [118] used a low $Ca^{2+}$ version of the SM7 medium, while Jang and Hwang [120] used a modified version of M4 medium.

Exposure time and organism age are also parameters that vary a lot in toxicological evaluation. All studies reported here used *D. magna* as the model organism, however, with different ages: < 24 h neonates, 5 days old, and 7 days old. Exposure times varied from 24 to 72 h of exposure. *D. magna* has a fast development, and the first brooding usually happens at around 10 days old, therefore the basic physiology of a 24 h neonate and a 7 day old daphnid are very different, thus the toxicological response may be different, and the same can be said for exposure time, the effects of an exposure of 24 h will be significantly different than those from a 72 h exposure.

Overall, it is clear that binary mixtures of NMs and pollutants may influence the toxicological outcomes differently compared to non-binary exposures. However, due to the intrinsic characteristics of NMs, the variability in the test guidelines and methodologies used strongly influences the comparability of the produced results. Also, due to the variability in NMs properties, extensive characterization is recommended to allow comparability of the results. Furthermore, *Daphnia* is a filter feeder, therefore is constantly moving in the water column, and thus the toxicity is going to be strongly influenced by whether the NM is in suspension, thus in order to fully understand the results it is important to consider not only the NMs properties, but also its colloidal stability under the exposure conditions and over the exposure duration.

### *Inorganic nanomaterials*

There is a wide range of inorganic NMs, and most of the studies evaluating co-exposure of NMs and contaminants address this class of materials. In this section, twenty-four studies were found (Table 2) addressing NMs of titanium dioxide ($TiO_2$), zinc oxide (ZnO NPs), silver (AgNPs), aluminium oxide ($Al_2O_3$ NPs), cerium dioxide

**Table 2**
Papers addressing the combined toxicity of inorganic NM and pollutants towards *Daphnia* model.

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Ref. |
|---|---|---|---|---|---|---|---|---|
| $Al_2O_3$ NPs | Purchased from Skyspring Size: 10 nmHydrodynamic size: 600–1500 nmSurface area:~600 $m^2$ $g^{-1}$ | $NaH_2AsO_4$ (As(V)) | EPA standard | $Al_2O_3$: 50–200 mg $L^{-1}$;As(V): 4.5; 3.6 and 1.5 mg $L^{-1}$ | Acute toxicity(48 h) Bioaccumulation | *C. dubia* < 24 h (neonate) | High As concentration: decreased toxicityHigh $Al_2O_3$ concentration: enhanced toxicity and accumulation of As(V). | Wang et al.[142] |
| $TiO_2$ NPs | Purchased from Skyspring Anatase 99%Size 5 – 10 nmHydrodynamic size: 0.6 – 2.5 μmZeta potential: 16Preparation: sol-gel | $NaH_2AsO_4$ (As(V)) | EPA synthetic hard water | $TiO_2$: 1–300 mg $L^{-1}$As(V): 0.45–4.5 mg $L^{-1}$ | Acute toxicity (24 h) | *C. dubia* < 24 h (neonate) | Co-expoure with $TiO_2$ NPs (non toxic concentration):Enhanced acute toxicity of As at low NP concentration.Decreased acute toxicity of As at high NP concentration. | Wang et al.[17] |
| $TiO_2$ NPs | Purchased from Nanjing High Technology MaterialAnatase | $Cu(NO_3)_2$ ($Cu^{2+}$) | Non described | $TiO_2$: 2 mg $L^{-1}$Cu: 10 – 100 ug $L^{-1}$ | Acute toxicity (72 h) BioaccumulationM-etallothionein | *D. magna*21–25 days (adults) | The mixture enhanced the acute toxicity & bioaccumulation, and decreased the level of metallothionein. | Fan et al.[138] |
| $CeO_2$ NPs$TiO_2$ NPs | Purchased from SkyspringNano-$CeO_2$Size: 10–30 nmHydrodynamic size: > 1000 nmSpecific surface: 30–50 $m^2$ $g^{-1}$Nano-$TiO_2$AnataseSize: 5–10 nmHydrodynamic size:30–60 nmSpecific surface: 50 $m^2$ $g^{-1}$ | Lead ($Pb^{2+}$)Standard solution | EPA standard | nano-$CeO_2$ ornano-$TiO_2$:120 and 200 mg $L^{-1}$Pb(II): 0–4.5 mg $L^{-1}$ | Acute toxicity | *C. dúbia* < 24 h (neonate) | NPs enhanced toxicity and accumulation of Pb. Acid medium (pH 6.8) enhanced overall Pb toxicity. | Hu et al.[143] |
| $TiO_2$ NPs | Purchased from Nanjing High Technology MaterialPreparation: chemical vapour deposition (CVD)AnataseHydrodynamic size: 189 – 266 nmSurface area:114.7 $m^2$ $g^{-1}$ | Cadmium ($Cd^{2+}$)Zinc ($Zn^{2+}$)radiolabeled | SM7 | $TiO_2$: 0.5; 1; 2 mg $L^{-1}$ | Biokinetics (Dietary assimilation efficiency and efflux rate constant) | *D. magna*~14 days (adults) | Adsorption is enhanced in the presence of $TiO_2$ NMs. | Tan et al.[140] |
| $TiO_2$ NPs | Three different types:1. P25 $TiO_2$ NPs / Evonic DegussaSize: ~30 nm/SSA: 47 $m^2$ $g^{-1}$2. Hombikat UV100 NPs / Sachtleben Chemie – Size < 10 nm / SSA: 288 $m^2$ $g^{-1}$3. LW-S $TiO_2$ NPs / Sachtleben ChemieSize: ~300 nm/SSA: 11.5 $m^2$ $g^{-1}$ | Cadmium ($Cd^{2+}$)Analytical grade(STD Cd 1000 ppm in 2% HNO3) | SM7 | $TiO_2$: 2 mg $L^{-1}$Cd: 10 – 640 ug $L^{-1}$ | Acute toxicity (48 h) Bioaccumulation | *D. magna* < 24 h (neonates) | Facilitated uptake of Cd by $TiO_2$ NPs due to food habit (filtering) of daphnids.No change in Cd toxicity. | Hartmann et al.[144] |
| CuO NPsZnO NPs | Purchase from Beijing NachenCuOSize 51 nmSurface area:80 $m^2$ $g^{-1}$ ZnOSize 48 nmSurface area:90 $m^2$ $g^{-1}$ | Mixture between NPs (CuO and ZnO NPs) | OECD 202 | Acute toxicity: 0.01–10 mg $L^{-1}$Chronic toxicity CuO: 0.0004–0.25 mg $L^{-1}$ZnO: 0.0008–0.5 mg $L^{-1}$Mixture ratio: 1:1 | Acute toxicity (48 h)Chronic toxicity(21 days) Semi -static | *D. magna* < 24 h | The mixture between NMs resulted in impairments at mortality, filtration rate, reproduction and body length increase. The mixture toxicity index (MTI) was calculated to confirm the synergistic toxicity profile in binary mixture. | Zhao et al.[145] |

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**Table 2** (*continued*)

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Ref. |
|---|---|---|---|---|---|---|---|---|
| $TiO_2$ NPs | Institute for Particle TechnologyAnatase: rutile: amorphous ratio 78:14:8Surface area of 50 ± 15 $m^2$ $g^{-1}$Hydrodynamic size: 86 ± 1.3 nm(PDI 0.118–0.164) | $AgNO_3$ ($Ag^+$)$Na_2HAsO_4$ (As (V))$CuSO_4$ and Cu ($NO_3$)$_2$ ($Cu^{2+}$) | OECD 202 | $TiO_2$: 2 mg $L^{-1}$;$AgNO_3$: 0.5–16 ug $L^{-1}$ $Na_2HAsO_4$:32000 ug-$L^{1}$ $CuSO_4$: 6–192 ug $L^{-1}$ Cu ($NO_3$)$_2$: 6–192 ug $L^{-1}$ | Acute toxicity (72 h) | *D. magna* < 24 h (neonates)~14 days (adults) | Ag: the presence of $TiO_2$ NPs increased toxicity.As and Cu: co-exposure with $TiO_2$ NPs reduced toxicity. | Rosenfeldt et al.[146] |
| $TiO_2$ NPs | $TiO_2$ A100Purchased from Crenox99% anataseSize 6 nmSurface area: 230 $m^2$ $g^{-1}$Hydrodynamic size: 87.3 nm$TiO_2$ P25Purchased from Evonik~75% anatase / ~25% rutileSize 21 nmSurface area: 50 $m^2$ $g^{-1}$Hydrodynamic size: 86 nmMKN-$TiO_2$-R050PPurchased from Mknano99% rutileSize 50 nmSurface area: 30 $m^2$ $g^{-1}$Hydrodynamic size: 90 nm | $Cu(NO_3)_2$ ($Cu^{2+}$) | ASTM Hard water | nano-$TiO_2$:A-100 = 1 mg $L^{-1}$ P25 = 2 mg $L^{-1}$R050-P: 4 mg $L^{-1}$C-u:384–768 μg $L^{-1}$ | Acute toxicity(48 h) | *D. magna* < 24 h | All nano-$TiO_2$ reduced Cu toxicity R050P = P25 > A100. Crystalline structure is more important than particle size (Anatase Cu adsorption better than rutile). Presence of Natural organic matter (NOM) and $TiO_2$ decreased Cu toxicity. | Rosenfeldt et al.[147] |
| C-AgNPs | Citrate coatedPurchased from ABC NANOTECH15 ± 3.59 nm (TEM)Hydrodynamic diameter:50 ± 0.7 nmZeta potential (pH 7):−21.17 mVDissolved fraction: 4.86% | $As_2O$ (As(V))$CdCl_2$ ($Cd^{2+}$)$CuSO_4$ ($Cu^{2+}$) | EPA Synthetic hard water | AgNPs: 10 ug $L^{-1}$;As (V): 1–50 mg $L^{-1}$;Cd: 0.1–5 mg $L^{-1}$;Cu: 1–50 mg $L^{-1}$ | Acute toxicity (24 h) Bioaccumulation | *D. magna*Acute toxicity: > 24 h (neonate) Bioaccumulation: 7 days old (adults) | As(V) and Cu: no change in acute toxicity / reduction of bioaccumulation. Cd: enhanced acute toxicity and bioaccumulation | Kim et al.[148] |
| ZnO NPsAgNPs | ZnO:Purchased from Nanosum Size 30 nmHydrodynamic size: 1061 nmZeta: −2.84 mVAgNPsPurchased form AmepoxSize 3 – 8 nmHydrodynamic size: 127 – 132 nm | $AgNO_3$ ($Ag^+$)$ZnCl_2$ ($Zn^{2+}$)+Mixture between NPs (ZnO and AgNPs) | ASTM Hard water | ZnO NPs: 0.25–5 mg $L^{-1}$ ZnCl20.1–3.2 mg $L^{-1}$ Ag NPs1 to 25 μg $L^{-1}$AgN-O30.5–10 μg $L^{-1}$ | Acute toxicity(24 and 48 h) Feed inhibition | *D. magna* < 24 h4–5 day old (feed inhibition) | Based on concentration addition (CA) and independent action (IA) models, the interaction could be classified as:ZnO + AgNPs = synergistic toxicity ZnO + $ZnCl_2$ = Antagonism.AgNPs + $AgNO_3$ (low ion concentration) = antagonism.AgNPs + $AgNO_3$ (high ion concentration) = synergistic. | Lopes et al.[133] |
| $TiO_2$ NPs | Purchased form Evonik. Size = 21 nm Surface area = 50 $m^2$ $g^{-1}$Anatase, Rutilo, amorphous ratio 78:14:8 | $Cu(NO_3)_2$ ($Cu^{2+}$) | ASTM Hard water | nano-$TiO_2$:200,000 μg $L^{-1}$ Cu: 1200–38400 μg $L^{-1}$ | Acute toxicity (48 h and 72 h) | *D. magna* < 24 h | Without aging: $TiO_2$ reduced Cu toxicity.With aging: $TiO_2$ increased Cu toxicity. | Rosenfeldt et al.[149] |
| $TiO_2$ NPs | Purchased from Sigma AldrichAnatase: 99.7%Average surface:50 $m^2$ $g^{-1}$Size 25 nmHydrodynamic size: 600 nmZeta potential:−0.71 mV | $Na_3AsO_4$ (As(V)) | SM7 | $TiO_2$: 2 and 20 mg $L^{-1}$ As (V): 5–50 μM | Acute toxicityBioaccumulation | *D. magna*7 days old | Decrease of As(V) toxicity in the presence of $TiO_2$. | Li et al.[150] |

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**Table 2** (*continued*)

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Ref. |
|---|---|---|---|---|---|---|---|---|
| PAA-$TiO_2$ NPs | Polyacrylate-coated $TiO_2$ NPs (74% of total weight) Size: 1 – 10 nm | Cadmium ($Cd^{2+}$) (does not say if it is salt or solution) | SM7 | $TiO_2$: 4 mg $L^{-1}$Cd: 10–300 μg $L^{-1}$ | Acute toxicity (24 h) Bioaccumulation in the presence of low and high concentration of Ca (0.2 and 20 μM) | *D. magna*7 days old | The presence of $TiO_2$ NMs reduced the toxicity of Cd at low Ca concentration, the $TiO_2$ NM uptake was by endocytosis and passive drinking. At high Ca concentration, the Ca reduced the stability of $TiO_2$ NMs. The NMs increase possibility their accumulation by active ingestion and Cd concentration on digestive tract. | Tan et al.[151] |
| $TiO_2$ NPs | Purchased from Sigma AldrichSize < 25 nm AnataseAverage area =45–55 $m^2$/g | Cadmium ($Cd^{2+}$)Zinc ($Zn^{2+}$)radiolabeled | SM7 | Nano-$TiO_2$ = 1 or 5 mg $L^{-1}$ | Metal uptake(32 h) | *D. magna* | The mixture of nano-$TiO_2$ and algae had the lowest metal uptake in Daphnia when compared to single nano-$TiO_2$ or algae exposure. The presence of $TiO_2$ reduces the metal bioaccumulation from algae, which may further in less metal accumulation in D. magna over time. | Tan et al.[152] |
| AuNPs | Purchased from Sigma-AldrichCitrate stabilized ~4.56 ± 0.23 nmSpherical particle: 96% | Microplastic (MP) Fluorescent red micro-spheresPurchased from Cospheric-innovations in MicrotechnologyDi-ameter: 1–5 μmDensity: 1.3 g $cm^{-3}$,Excitation peak at 575 nm and emission peak at 607 nm | ASTM Renewal daily | AuNP: 0.2 or 2 mg $L^{-1}$MP: 0.02 or 0.2 mg $L^{-1}$ | 21 days chronic toxicity: mortality, somatic growth and reproduction | *D. magna* < 24 h (neonate) | At low concentrations of AuNPs and MPs: antagonism at mortality endpointAt high concentrations of AuNps and MPs: synergism at mortality endpointThe chronic exposure to AuNPs, MP, and their mixtures can impair development and reproduction endpoints, but the type of interaction could not be classified. | Pacheco et al.[153] |
| $TiO_2$ NPs | Purchased from Ishihara Sangyo KaishaHydrophilic and hydrophobicSize: 29.5 and 36.7 nmSurface area: 47.5 and 38.5 $m^2$ $g^{-1}$Zeta potential: −28 and −6 mV | Copper ($Cu^{2+}$) (does not say if it is salt or solution) | SM7 | Concentration not shown | Acute toxicity (48 h)ROS | *D. magna*14 days (adults) | Hydrophilic and hydrophobic $TiO_2$ NMs enhance mortality due to $Cu^{2+}$. | Liu et al.[154] |
| ZnO NPsTiO$_2$ NPs | ZnO NPs: < 50 nm / Alfa AesarTiO2: < 100 nm / Sigma-Aldrich | $AgNO_3$ ($Ag^+$) | ISO standard freshwater | $AgNO_3$: 0.5–2.5 ug $L^{-1}$ZnO: 38–20000 ug $L^{-1}$$TiO_2$: 781–200000 ug $L^{-1}$ | Acute toxicity (48 h) | *D. magna* < 12 h (neonate) | The sum of toxic unit (STU) index was used to classify the mixture.Synergistic effect between the NMs (ZnO and $TiO_2$) in $Ag^+$ mixture was observed based on mortality endpoint. | Park et al.[155] |

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**Table 2** (*continued*)

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Ref. |
|---|---|---|---|---|---|---|---|---|
| $TiO_2$ NPs | Purchased from AcrosAverage area = 50 $m^2g^{-1}$Size = 21 nm | $NaAsO_2$ (As(III)) $Na_2HAsO_4$ (As(V)) | SM7 | Nano-$TiO_2$ = 1 mg $L^{-1}$As(III) and As(V) = 0.2 mg $L^{-1}$ | Transgenerational (21 days) | *D. magna* < 24 h (neonates)~14 days (adults) | The binary mixture exposition during parental generation alleviate the impairments on the health of offspring (mortality and reproduction inhibition). Moreover, this effect was more prominent with As(V) exposition than that of As(III). | Fan et al.[156] |
| $TiO_2$ NPs | Purchased from Skyspring Nanomaterials Inc. Size: 5–10 nmAnatase,99% | $Pb(NO_3)_2$ ($Pb^{2+}$) | EPASynthetic water moderate hardness | Nano-$TiO_2$ = 0–1200 mg $L^{-1}$Pb = 0 – 2500 ug $L^{-1}$ | Acute toxicity(24 h) with and without food (algae) Bioaccumulation | *C. dubia* < 24 h (neonates) | Nano-$TiO_2$ elevated the Pb toxicity. The presence of algae reduced the mortality and Pb uptake. | Liu et al.[157] |
| Iron oxide NPs | PVP-$Fe_3O_4$ NPsLiquid type Iron (II, III) oxide < 25 nmCoated w/ PVPPurchased from NanoComposix | $ZnSO_4$ ($Zn^{2+}$) | ISO Standard freshwater | PVP-$Fe_3O_4$ NPs0.16–160 ug $mL^{-1}$$ZnSO_4$:0.08–20 ug $mL^{-1}$ + Mixture | Acute toxicity (48 h) | *D. magna* < 15 h (neonate) | Concentration addition (CA) and effect addition (EA) were applied for classified the interaction between components in binary mixtures (PVP-$Fe_3O_4$ NMs and $ZnSO_4$). The antagonism effects was observed with increase in the total concentration of $ZnSO_4$ and PVP-$Fe_3O_4$. | Park et al.[158] |
| Iron-modified biochar | Biochar was prepared from pyrolysis of giant Miscanthus (4-cm long) and modified with ferric nitrate | $NaAsO_2$ (As(III)) | ISO medium | Leaching solution of interaction 2.0 g $L^{-1}$ Iron-modified biochar powder or 2.0 g $L^{-1}$ Iron-modified biochar beads and 20 mg $L^{-1}$ As(III) | Acute toxicity (24 h) | *D. magna*Neonate | The As (III) concentration leached from interaction with NM (< 2.0 mg $L^{-1}$) should not induce acute toxicity. However, 10% immobilization was observed at 1 mg $L^{-1}$ As (III) in the leachate with interaction with Fe-biochar powder. which was possibly due to the uptake of As(III) adsorbed onto NMs by the daphnids. | Kim et al.[159] |
| ZnO NPs | Purchased from Alfa Aesar Size < 50 nmHydrodynamic diameter in medium: 730.3–1149 nmZeta potential: −12.3 to −1.7 mV | $AgNO_3$ ($Ag^+$) | ISO standard | ZnO: 0.04–20 $mgL^{-1}$ $AgNO_3$: 0.5–2.5 $ugL^{-1}$ | Acute toxicity (48 h) Bioaccumulation | *D. magna < 15 h (neonate)* | In higher ratio of ZnO NPs to $AgNO_3$ concentrations, co-exposure with ZnO NPs showed synergism effect;In lower ratio of ZnO NPs to $AgNO_3$, antagonism effect was observed. | Baek et al.[141] |
| Black-$TiO_2$ NPs | Synthetized by impregnation method with urea ($CH_4N_2O$) and P25 $TiO_2$AnataseSize > 100 nm | CiprofloxacinCIP $C_{17}H_{18}FN_3O_3$ | OECD 202 | Non described | Acute toxicity (96 h) | *D. magna* < 24 h (neonates) | The products of degradation produced by the presence of black-$TiO_2$ under visible LED light irradiation was less toxic than the untreated controls (only $TiO_2$ without LED) due to the decomposition of CIP and generation of the simpler intermediate products with less toxicity. | Sarafraz et al.[160] |

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**Table 2** (*continued*)

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Ref. |
|---|---|---|---|---|---|---|---|---|
| ZnO-NMAg-NPZnO/Ag-NS | Synthesized byLaser Assisted Flow Deposition and Pulsed Laser Ablation. Size:ZnO-NM (tetrapod): 40–50 nmAg-NP (spherical): 5–12 nm)ZnO/Ag-NS (nano-heterostructure): tetrapod of zinc oxide decorated with silver nanoparticles (1–3% mol. Ag.). | Mixture between ZnO-NM andAg-NP | OECD 202 and 211 | Nominal concentrations ranged from 0.5 to 1.3 mg·Zn·$L^{-1}$ for theZnO-NM treatments, 0.05–0.25 mg·Ag·$L^{-1}$ for Ag-NM and 0.13–0.63 mg·ZnO/Ag·$L^{-1}$ (1–3% mol. Ag) for ZnO/Ag-NS. | Acute toxicity (48 h) Reproduction (21 days) | *D. magna* < 24 h (neonates) | ZnO/Ag-NS showed higher toxicity thanthe predicted based on individual toxicity.ZnO/Ag-NS did not present the samebehavior of a mixture of ZnO-NM andAg-NPZnO/Ag-NS should be considered as a single material showing implications for regulation of nano-heterostructures. | Azevedo et al.[161] |

($CeO_2$ NPs), copper oxide (CuO NPs), iron oxide (PVP-$Fe_3O_4$ NPs), AuNPs and iron-modified biochar. All studies analysed binary mixtures of the NMs with metal ions, such as, $Ag^+$; As(III), As(V), $Cu^{2+}$; $Cd^{2+}$; $Zn^{2+}$; $Pb^{2+}$ and with microplastics.

In the aquatic environment, a NM's behaviour is affected by physical and chemical processes, such as homo/hetero agglomeration, sedimentation, photochemical reaction, dissolution, redox reactions, coating degradation, and biomolecule/macromolecule binding [67]. These processes strongly depend on the NMs' physical-chemical properties, such as size, shape, surface area, charge, as well as the environmental characteristics, such as pH, ionic strength, NM concentration, specific surface area (SSA) and the presence of macromolecules (e.g. NOM and/or proteins) [67]. The combination of these influences NMs dissolutions rates, cellular uptake, and reactivity, modulating their toxicity profile [130].

Dissolution plays a crucial role in inorganic NM toxicity. If the NMs dissolve in the surrounding medium, the uptake mechanism and the biological response to released ions could be distinct from the responses to the NMs themselves [131]. For example, Ponyton et al. observed a disruption at gene expression level of protein metabolism and signal transduction in *D. magna* upon AgNPs exposure, while $AgNO_3$ caused a downregulation of developmental processes, especially sensory development. Thus, both the NM and its dissolved ions will contribute to the biological response in the studies[132]. To simulate the toxicity under the dissolution scenario, Lopes and coworkers [133] exposed *D. magna* to mixtures of ZnO and AgNPs and their respective ionic counterparts ($ZnCl_2$ and $AgNO_3$). Interestingly, they observed that the ratio of $Ag^+$ ions and AgNPs influenced the toxicological response. When the concentration of $Ag^+$ (from $AgNO_3$) increased, they observed a synergistic effect while antagonism occured when AgNPs were dominant in the mixture (low $Ag^+$). Thus, knowledge of dissolution kinetics of metallic NMs is fundamental to understanding the toxicity of metallic NMs as it will co-occur with the dissolved ions. To this end, some analytical techniques (as ultrafiltration, sp-ICP-MS, etc.) could be applied to measure the free ions in the NMs dispersion.

Moreover, once NMs enter the environment, they will inevitably interact with co-existing contaminants. The high reactive and large surface area allows the interaction and/or surface complexation through electrostatic interactions [134]. In addition, this interaction could facilitate the degradation of different compounds over time. For example, $TiO_2$ NPs are extensively applied in water treatment to facilitate the degradation of organic pollutants and the reduction of metals to their zero oxidation states [135]. This process happens due to the photocatalytic splitting capability of $TiO_2$ NMs in aqueous solution under UV light. The photodegradation could lead to the formation of toxic products [136], such that the intermediate products resulting from this process could show higher toxicity profiles then the pristine chemicals and thus needs to be carefully evaluated.

The main mechanism of interaction between inorganic NMs and environmental components is adsorption, which was analysed for most studies addressed here. The modification of the toxicological outcomes by the adsorption of contaminants to NMs can go in two ways; NMs can facilitate the delivery of contaminants, a process known as the trojan horse effect, where the NM acts as a carrier increasing the uptake by organisms [4], or NMs can reduce the concentration of contaminants in the environment either by the strong adsorption or aggregation and/or sedimentation, decreasing the co-contaminant mobility and bioavailability [137]. Mixture studies involving NMs need to consider both possible outcomes, as shown schematically in Fig. 3.

Trojan horse effects were observed in several co-exposure studies, where the presence of NMs led to increased bioaccumulation and toxicity of contaminants in daphnids. For example, Fan et al. observed that the co-exposure of copper ions ($Cu^{2+}$) with $TiO_2$ NMs (nontoxic NM concentration of 2.0 mg $L^{-1}$) increased *D. magna*

mortality, by decreasing the $LC_{50}$ (the concentration that kills half of the population) of $Cu^{2+}$ from 111 $mgL^{-1}$ to 42 $mgL^{-1}$ [138]. The co-exposure resulted in increased $Cu^{2+}$ bioaccumulation in daphnids, reaching 18–31% higher than during exposure to metal ions only, which can be explained by the high adsorption capacity of $TiO_2$ NPs for $Cu^{2+}$. The complexation of $Cu^{2+}$ onto the $TiO_2$ MPs decreases the concentration of free metals ions in the *D. magna* medium. On the other hand, due to ingestion of the copper complexed with $TiO_2$ NMs, the co-exposure enhanced the accumulation of $Cu^{2+}$. Moreover, co-exposure reduced the induction of metallothionein, an important enzyme for metal detoxification, probably due the competition between the NMs and $Cu^{2+}$ for sulfhydryl groups, inhibiting this mechanism and contributing to an increase in the combined toxicity (additive interaction).

According to Weltens et al., the uptake and bioaccumulation of metals in daphnids could be related to the desorption of these contaminants inside the organism gut, which is a complex environment that presents high enzymatic activity and low pH [139]. Tan et al. studied this mechanism by analysing the dietary assimilation efficiency and efflux rate of two toxic metals (radiolabelled $Cd^{2+}$ and $Zn^{2+}$) combined with $TiO_2$ NMs [140]. They observed that daphnids could actively ingest the $TiO_2$ NMs with contaminants adsorbed, which were desorbed within the gut. This knowledge points to the fact that NMs could act as a carrier of xenobiotics into daphnids and deliver these inside the digestive tract thus increasing the potential risks of contaminants accumulation.

Moreover, the Trojan horse effect could be influenced by the exposure conditions. For example, Baek et al. studied the bioaccumulation and acute toxicity of mixtures of ZnO NPs and $AgNO_3$ ($Ag^+$) in *D. magna* under different concentration ratios [141]. At a high concentration of ZnO NPs, the co-exposure enhanced the silver bioaccumulation and daphnid mortality, due to the presence of ionic Zn (released from ZnO NPs) and/or by ZnO NPs acting as carrier for $Ag^+$ (Trojan horse effect). On the other hand, at lower ZnO NP concentrations a reduction in $Ag^+$ bioaccumulation and toxicity were observed, which may be due to $Zn^{2+}$ and $Ag^+$ competition for receptor binding sites on *Daphnia*, leading to an antagonistic effect.

Similarly, Rosenfeldt et al. observed the influence of a mixture of NMs ($TiO_2$) and three different metal ions (As(V), $Cu^{2+}$ and $Ag^+$) [146]. The bioaccumulation and toxicity of As(V) and $Cu^{2+}$ was reduced in co-exposure with $TiO_2$ NPs in *D. magna*. In the medium, the NMs rapid agglomerated, as observed by the increase of hydrodynamic size during the time, and sedimented together with the adsorbed As(V) and $Cu^{2+}$. This process led to a lower concentration of $TiO_2$ NPs and free toxic metals in the medium and, consequently, reduced their bioavailability to filter feeders. However, the same study observed a synergistic effect on toxicity and bioaccumulation in co-exposure with silver ($Ag^+$) probably due to the desorption of $Ag^+$ in the gut (Trojan horse effect).

Agglomeration seems to play an important role in decreasing the toxicity of NMs and contaminants in co-exposure outcomes. Ionic forces in the aquatic environment can affect colloidal stability due to electrostatic screening of NM charge, causing agglomeration and sedimentation. Thus, in a high ionic strength environment, NMs show low colloid stability, especially in the presence of divalent cations [162]. Furthermore, low colloidal stability leads to an increase of NM size. This process could modify the available surface area with which NMs can interact with other contaminants, and affect their ability to be internalized, and subsequent interactions with organisms and bioavailability in the water column [163]. In this way, the colloidal behaviour of NMs plays a key role in their toxicity profile, transport, and fate [130].

For example, Tan et al. observed the influence of colloidal stability on the toxicity profile of $TiO_2$ NPs and cadmium ($Cd^{2+}$) in two different scenarios: low and high calcium concentration in the medium, and observed that colloidal stability influences the NM uptake route in *D. magna* [151]. In both scenarios, the co-exposure with NPs reduced the toxic outcome of $Cd^{2+}$. However, the uptake route of $TiO_2$ agglomerates was different, as observed by X-ray fluorescence microanalysis (μXRF). This technique allows analysis of the presence of major, minor and trace elements in microscopic sample surface areas and generation of a 2D spatial map of the distribution of chemical elements [164]. At low concentration of $Ca^{2+}$, $TiO_2$ NPs showed better colloidal stability and lower agglomerate size. The metal-adsorbed NPs were internalized by endocytosis via the cells of the filtration apparatus and passive drinking. In this scenario, metal accumulation was observed to be well distributed throughout the organism. However, at higher salt concentration, the NPs showed lower colloidal stability which led to increased agglomerate size in the medium, causing metal accumulation in the abdominal zone (Fig. 7). Therefore, ingestion of NM agglomerates is the principal route of $Cd^{2+}$ uptake. Under this condition, $Cd^{2+}$ accumulation was enhanced. Hence, the NM agglomeration state could modify completely the metal bioaccumulation upon combined exposure.

Moreover, the concentration of NMs modifies their colloidal behaviour and toxicity. For example, Park et al. studied the acute toxicity and bioaccumulation in *D. magna* exposed to a mixture of PVP-$Fe_3O_4$ NPs and zinc sulphate ($ZnSO_4$) at different mixing ratios [158]. Based on the dose-response curves, the mode of action of the mixture was predicted by conventional models for binary toxicity using the theoretical models of concentration addition (CA) and independent action (IA) index. They observed that low concentration-combinations (< 5.0 μg $mL^{-1}$ of $ZnSO_4$ and < 9.6 μg $mL^{-1}$ of PVP-$Fe_3O_4$ NPs) caused a $ZnSO_4$ dose dependent synergistic effect. Nevertheless, at high concentration-combinations (> 5.0 μg $mL^{-1}$ of $ZnSO_4$ and > 9.6 μg $mL^{-1}$ of PVP-$Fe_3O_4$ NPs), the binary mixture shows an antagonist effect. The authors suggested that these effects were dominated by the high adsorption capability and low stability of PVP-$Fe_3O_4$ NPs at high concentrations leading to sedimentation of the NPs with metal adsorbed, reducing its availability to daphnids and thus its toxicity.

NM concentration effects were also observed by Wang et al. studying the toxicological effects of a non-toxic concentration of $Al_2O_3$ NPs co-exposed with As(V) to *C. dubia* [142]. The NM shows no toxicity until 200 mg $L^{-1}$ over 24 h. On the other hand, the toxicity enhancement of As(V) is concentration dependent and the $LC_{50}$ was 3.6 mg $L^{-1}$. In co-exposure, low $Al_2O_3$ concentration (1.0 mg $L^{-1}$) had almost no effect on the metal toxicity and the $LC_{50}$ calculated was close to that from single exposure of As(V). Conversely, increasing the dose of $Al_2O_3$ to between 20 and 100 mg $L^{-1}$, resulted in a significant increase in toxicity and bioaccumulation and the $LC_{50}$ calculated was 1.0 mg $L^{-1}$. Furthermore, the uptake of As(V)-loaded nano-$Al_2O_3$ played a very important role in the toxicity response of *C. dubia*, pointing to a Trojan horse effect.

Similarity, $TiO_2$ NPs have the potential to adsorb As(V) onto its surface. Thus, a reduction of the residual As(V) concentration in the medium was observed. Consequently, the presence of NM impacts the mixture toxicity. At low $TiO_2$ concentrations (less than 50 mg $L^{-1}$) the toxicity of As(V) is significantly increased. In this scenario, the sorption of As(V) onto the $TiO_2$ NMs positively contributed to overall toxicity once NMs enter daphnids bodies by oral uptake as “fake food”. However, with increasing NMs concentration (100 and 200 mg $L^{-1}$), the As(V) toxicity is decreased. Colloidal stability plays an important role in this result, as increasing the $TiO_2$ NP concentration led to NM agglomeration, reducing the bioavailability of $TiO_2$ NP/As(V) complexes, decreasing their oral uptake by *C. dubia*, and consequently bioaccumulation and toxicity [17].

Another medium characteristic that could modulate the NMs behaviour and the toxic outcome is the pH. To observe this influence, Hu et al. studied the impacts of pH on lead ($Pb^{2+}$) toxicity in a binary mixture with $CeO_2$ NPs or $TiO_2$ NPs using *C. dubia* as the model

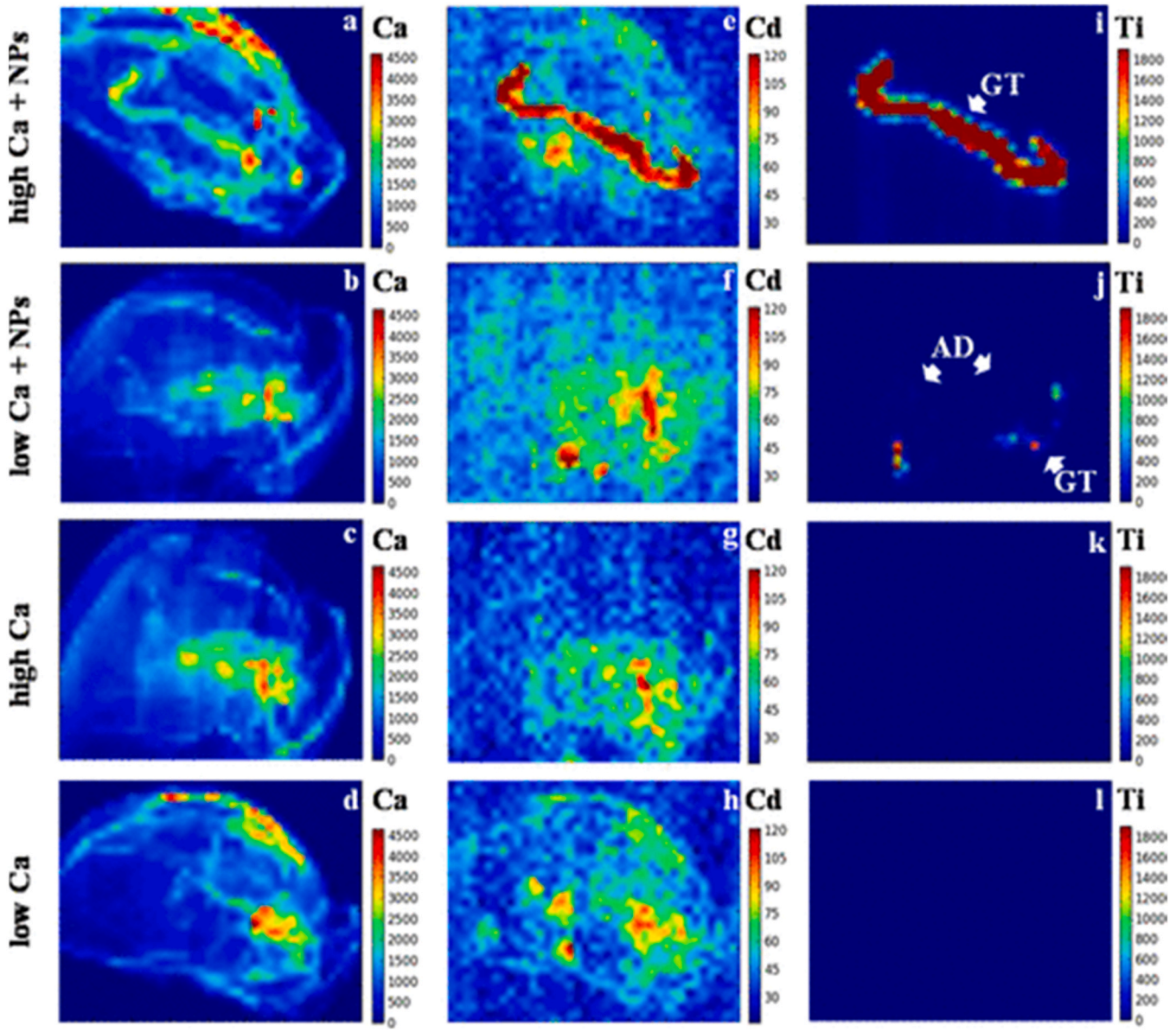


**Fig. 7.** Quantitative spatial distribution of $Ca^{2+}$ (a–d), $Cd^{2+}$ (e–h) and $TiO_2$ NPs (i–l) in *D. magna* neonates exposed for 24 h as determined by X-ray fluorescence microscopy (μXRF). The daphnids were exposed to $Cd^{2+}$ (100 μg $L^{-1}$) in the presence (a, b, e, f, i, and j) or absence (c, d, g, h, k, and l) of $TiO_2$ NPs (4 mg-Ti $L^{-1}$) at low (b, f, j, d, h, and l) and high (a, e, i, c, g, and k) $Ca^{2+}$ medium concentrations. The arrow indicates the gut (GT) or abdominal (AD) area (reprinted with permission from ref. [131], Copyright 2021 American Chemical Society).

organism [143]. Both particles showed high $Pb^{2+}$ adsorption capability, however, this capacity was higher for $TiO_2$ NPs, as evidenced by Energy Dispersive X-Ray Spectroscopy (EDS) analysis, which showed an increase of hydrodynamic size and changes in zeta potential (i.e., surface charge). Overall, co-exposure resulted in higher metal bioaccumulation and acute toxicity. However, due to its high adsorption capability the co-exposure with $TiO_2$ NP caused a more pronounced bioaccumulation and toxicity effect than $CeO_2$ NP. Hence, on reducing pH (from 7.8 to 6.8) an enhancement of overall toxicity was observed (in single and combined exposures) due to $Pb^{2+}$ speciation in solution and/or modified charges on the NP surface, enhancing its bioavailability in the water column and modulating the toxicological outcome.

In natural water bodies, the presence of food is a factor that also could modify the toxicity outcome. Considering more realistic exposure scenarios, Liu et al. studied the co-exposure of $TiO_2$ and $Pb^{2+}$ in *C. dubia* and the influence of algae (*Raphidocelis*) [154]. The $TiO_2$ NPs act as an excellent sorbent of $Pb^{2+}$ and the presence of 200 mg $L^{-1}$ $TiO_2$ NMs reduces free $Pb^{2+}$ in the medium from the soluble amount of 350 μg $L^{-1}$ to less than 10 μg $L^{-1}$. This interaction affects the toxic outcome of $Pb^{2+}$ to *C. dubia*. In combined exposure, the $TiO_2$ NPs act as a metal carrier, enhancing bioaccumulation and toxicity. Moreover, the influence of food (algae) in the co-exposition was explored to provide a more realistic scenario, and the presence of algae modified the intake of toxic metal, reducing the mortality from 80% to 35%, and decreasing the total $Pb^{2+}$ content in the daphnids. Hence, food may mitigate the toxicological effects in aquatic environments, both by reducing NMs and co-pollutant uptake and through the provision of extra energy that can be diverted to mitigating toxicity impacts.

The physical-chemical properties of NMs also contribute to mixtures toxicological profiles via unique mechanisms. For example, Liu et al. studied two different types of $TiO_2$ NPs (hydrophilic and hydrophobic) mixtures with $Cu^{2+}$ [154]. They observed enhanced metal toxicity with both hydrophilic and hydrophobic $TiO_2$. Nevertheless, despite similar lethality, the mechanisms involved were

different. The mixture with hydrophilic $TiO_2$ damaged the intestinal membrane more severely as observed by Scanning Electron Microscopy (SEM) images and enhanced $NA^+/K^+$ ATPase activities. On the other hand, the co-exposure with hydrophobic $TiO_2$ NPs showed higher bioaccumulation of $Cu^{2+}$ and high oxidative stress injury.

Moreover, Rosenfeldt et al. compared the toxicity of copper ($Cu^{2+}$) in binary mixtures with three different crystalline phases of $TiO_2$ NPs (anatase, rutile and anatase/rutile mixture) using *D. magna* [147]. $TiO_2$ NPs showed a high capacity for $Cu^{2+}$ adsorption, with rutile $TiO_2$ and anatase $TiO_2$ NPs decreasing by 80% the $Cu^{2+}$ residual in the water column, while the rutile/anatase $TiO_2$ NP reduced it by 60%. All three particles decreased $Cu^{2+}$ toxicity, however the anatase $TiO_2$ NPs mixture showed the lowest toxicity values, while rutile and rutile/anatase mixtures had similar toxicity values. The anatase $TiO_2$ NP structure showed higher porosity and binding sites (unsaturated oxygen and deprotonated surface hydroxyls) than the other crystalline phases, which may explain the toxicity results as the $Cu^{2+}$ ions may remain bound to the anatase $TiO_2$ following internalization. Besides crystalline phase, this study also evaluated the influence of NOM on $TiO_2$ NP $Cu^{2+}$ mixture toxicity, showing that NOM modifies the surface of $TiO_2$ NMs by increasing their zeta potentials, and as a consequence the $TiO_2$ NP adsorption capacity was increased, resulting in a reduction in toxicity for all three $TiO_2$ NPs as the $Cu^{2+}$ ions remained attached to the NPs following uptake rather than desorbing and inducing toxicity.

Similar to their interaction with other chemicals, NPs can adsorb NOM (e.g., humic and fulvic acids) and other macromolecules such as secreted proteins and polysaccharides onto their surface (eco-corona formation). This process may affect the NM toxicity due to modification of colloidal stability, alteration of dissolution behaviour, complexation with free metallic ions and changes to the surface that alter the NMs' interactions with pollutants [165]. Moreover, eco-corona formation modifies the NM surface providing a biological identity to the NMs which changes the way that organisms interact with it and, consequently, its toxicity [103,166,167]. As these macromolecules naturally exist in the environment, consideration of their presence is especially important to reach a more realistic exposition scenario, since NMs will never exist in the environment without instantaneously acquiring an eco-corona.

Not only the physical-chemical properties of NM influence their interaction with contaminants, but the characteristics of the pollutants also play a role. For instance, Kim et al. observed distinct results when AgNPs were co-exposed with toxic metals [148]. They observed that $Cu^{2+}$ presented good adherence onto the NPs due to the difference of charge. The mixture with As(V) was more competitive due to the negative charge. The daphnids survival was not affected by the addition of NMs in both cases (As (V) and $Cu^{2+}$) when compared to the metals exposition alone, while bioaccumulation significantly decreased since the interaction between metals and NMs reduced the bioavailability of this metals. In contrast, in combination with $Cd^+$, they observed an increase of toxicity and bioaccumulation of NMs-complexed Cd. The interaction between AgNPs and divalent ions present in the medium ($Ca^{2+}$) can lead to enhanced concentrations of intracellular $Ca^{2+}$, disturbing cell homeostasis, consequently stimulation of the $Ca^{2+}$ channel can increase $Cd^{2+}$ bioaccumulation since the uptake pathway of $Cd^{2+}$ uses this channel.

Azevedo et al. (2017) performed a mixture toxicity study on *D. magna* using zinc oxide tetrapods (ZnO-NM), a spherical silver nanoparticle (Ag-NP) and zinc oxide tetrapods decorated with 1–3% mol. of Ag NPs (ZnO/Ag-NS). The aim of this work was to understand if it is possible to predict the toxicity of combined nanostructures based on the toxicity of its isolated materials according to the CA conceptual model for predicting binary mixture toxicity effects. Importantly, the mixture of ZnO-NM and Ag-NP did not show an additive pattern but rather deviations such as dose-level and synergism, while Zn/Ag-NS showed higher toxicity when compared with the predicted toxicity based on the results from the individual materials. These results point out implications for regulation of nano-heterostructures, suggesting that these new hybrid materials need to be addressed as single materials and not only considering the toxicity of isolated component materials [161].

### *Polymeric nanomaterials*

Polymeric NMs are a large group of materials, they can be micelles, vesicles, star polymers, and inorganic-polymer hybrids of different shapes and sizes, different surface chemistry, surface charge, etc. Due to these numerous different types, a wide number of potential applications have been suggested, from environmental to nanomedicine [168,169]. As the number of polymeric NMs produced grows, it is inevitable that they will reach the aquatic environment. In this section, five studies were found (Table 3) addressing co-exposure of pollutants with polymeric NMs.

A recent study by Lin et al. quantitatively looked into the effects of the combined acute toxicity of nano-polystyrene (100 nm) and polychlorinated biphenyls (PCBs) on *D. magna* based on analytical chemical speciation by measuring the sorption coefficients of PCBs to nano-polystyrene (PS) [170]. Their findings showed that the toxicity of the combined chemicals depended on the relative concentrations of PCB and PS. When PCB was combined with a certain amount of PS, it was less toxic towards *D. magna*, while the toxicity was increased when using excessive amounts of PS. In this study they applied a passive dosing method to analyse the sorption coefficients of 8 solid chemicals [PCB-1, 3, 9, 11, 18, 77, hexachlorobenezene (Hexa-CB) and pentachlorobenzene (Penta-CB)] with 100 nm PS particles and correlated the speciation results with the observed toxicity endpoints, identifying joint toxicity effect of PS and PCB-18 to *D. magna*. For the mixture toxicity experiment they monitored the lethality to *D. magna* for 48 h by exposing the daphnids to a fixed concentration of PCB-18 (640 $\mu g\,L^{-1}$, i.e. the LC50 of PCB-18) and varying the PS concentration from 0 to 75 $mg\,L^{-1}$. Their results showed that when *D. magna* were exposed to PS in concentrations that are lower than 1 $mg\,L^{-1}$, their lethality decreased as the concentration of PS increased. When the PS concentrations were higher than 1 $mg\,L^{-1}$, the lethality of *D. magna* increased with the addition of PS particles. To interpret their results, the authors studied the sorption coefficient to understand the combined toxicity of PCB on 100 nm PS, and the results indicated that combining PCB-18 with PS can reduce the free concentration of organic pollutants and thus the toxicity towards *D. magna*.

Another study by Lin et al. revealed for the first time the effects of combining two complex matrices (nano-sized polystyrene (PS) and humid acid (HA)) on the bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in *D. magna* [171]. The aim of this study was to evaluate the joint effects of dissolved organic matter (DOM) and NMs on the bioaccumulation of typical PAHs (e.g., anthracene, phenanthrene, pyrene and others) by applying a modified matrix-inclusive biodynamic model with full quantification for determining the uptake pathways of PAH for various complex systems under environmentally realistic conditions and concentrations. For simulating the open water scenarios, where PAHs are present from various infinite sources, they utilized passive dosing vials, which allowed identification of the uptake pathways by enabling constant concentrations of freely dissolved PAHs during the entire experiment. Suspensions of four different matrices for PAH exposure were prepared using the (M7) artificial culture medium: the mixture of 100 $mg\,L^{-1}$ HA and 1 $mg\,L^{-1}$ PS; 100 $mg\,L^{-1}$ HA; 1 $mg\,L^{-1}$ PS, and a solution of M7 medium as a control. The results showed that the rate of PAH ingestion of the HA-PS or the HA matrix was faster than the PS matrix, which could be due to the variation in matrix mass added at the beginning of the study. The transfer of anthracene from the HA-PS, HA and PS matrices via the gut to lipids were analysed and

**Table 3**
Papers addressing the combined toxicity of polymeric NMs and pollutants towards *Daphnia* model.

| Nanomaterial | Characteristics | Joint with | Media | Concentration range | End-points | Specie / Age | Observed effects | Paper |
|---|---|---|---|---|---|---|---|---|
| Nano-polystyrene | (PCMs) were purchased from J &K Scientific Ltd. (Shanghai, China). 100 nm PS were purchased from Aladdin (Shanghai, China). | Polychlorinated biphenyls (PCBs) | OECD test guideline 202 | Constant concentration of PCB-18 ($LC_{50}$ = 640 μg $L^{-1}$) with different concentrations of 100 nm PS (0, 0.01, 0.1, 1, 5, 20, 40, 50, 75 mg $L^{-1}$) | Acute immobilization ($LC_{50}$) 48 h | *D. magna* < 24 h(neonate) | Combined exposures decreased the toxicity of PCB, but when adding excessive amounts of PS lethality was increased. | Lin et al.[170] |
| Polystyrene (PS) | 100 nm PS (2.5% w/v) were purchased from Aladdin (Shanghai, China). | Solid powder of HA (fulvic acid ≥90%) and Six unsubstituted PAHs | Artificial medium (M7) OECD standards 202 | HA (100 mg $L^{-1}$) with organic carbon concentration equivalent to 41 mg $L^{-1}$). Polystyrene (1 mg $L_{-1}$). The free concentration of PAHs in the exposure solutions was dynamically constant during the experiment. | Bioaccumulation experiments (0, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 14 h, 20 h, 28 h, and 36 h) | *D. magna* (12 days old) | The kinetics of uptake were most affected by the presence of complex matrix while the equilibrium of bioaccumulation was not affected by the matrix. | Lin et al.[171] |
| Polystyrene (PS) | Purchased as suspensions with concentrations of 2.5% w/v. | Phenanthrene | M4 medium | EC50 of NPs (0, 2.5, 5, 8.5, 11, and 14.5 mg $L^{-1}$). EC50 of Phe (0, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.2 mg $L^{-1}$) | For acute toxicity (48 h)For bioaccumulation (14 days incubation test) | For acute toxicity (neonates < 24 h old *D. magna*). For bioaccumulation (1 week old *D. magna)* | 10-μm MPs did not significantly affect the transformation, dissipation and bioaccumulation of phenanthrene, while the 50-nm NPs showed a significant effect, enhancing the bioaccumulation of phenanthrene-derived residues in the body of *D. magna* | Ma et al.[172] |
| Polystyrene (PS) Polyethylene (PE) | 300 nm PS were purchased from Thermo Fisher Scientific (Bleiswijk, the Netherlands) as liquid suspension 0.600 nm PS were purchased from Microparticles GmbH Forchungs (Berlin, Germany) as liquid suspension300–900 nm Polyethylene (PE) materials were purchased as powder from Cospheric LLC (Santa Barbara, CA, US) | Suwannee River NOM (1R101N)1000 mg $L^{-1}$ $Ag^{+}$ standards | Elendt M7 medium | NPD (1 mg $L^{-1}$), $Ag^{+}$ (0, 1, 2, 5, 10 μg $L^{-1}$) DOM (0, 1, 10, 50 mg $L^{-1}$) | Acute immobilization (72 h)Sorption of Silver ions onto NPDSublethal Toxicity | *D. magna* 9 days old | 600 nm PS absorb more Ag+ than the 300 PS and PE.The presence of the 300 nm PS and PE makes the Ag+ more toxic than when the 600 nm PS are present.When DOM are present, the sorption of Ag+ into the 600 nm PS decreased, while sorption into the 300 nm PE and PS increased. | Abdolahpur et al.[173] |
| Polystyrene (PS) | PS NPs were synthesized via emulsion polymerization of the styrene monomer (72.8 ± 6.8 nm) | Gly (N-(phosphonomethyl) glycine 96%) | *D. magna* cultured in M4 medium.Acute immobilization tests performed in ISO medium. | PS NPs (15.6, 31.2 62.5 125, 250 and 500 mg $L^{-1}$). Gly (6.2, 12.5, 25, 50, 100 and 200 mg $L^{-1}$) | Acute immobilization (48 h). Swimming behaviour. Chronic toxicity (21 days). ROS (48 h). Multigenerational (48 h). | *D. magna*For Acute, chronic and multigenerational (< 24 h)For ROS (9 days old *D. magna*) | Toxicity of Gly and PS NPs increases when they are present as a mixture.The swimming activities were decreased and the ROS production and immobility of daphnids were increased when exposed to the mixture compounds of Gly and PS NPs.The toxicity of individual and mixture compounds is transferred to future D. magna generations and induces reproduction effects. | (Nogueira et al.[174] |

the uptake kinetics of anthracene had the same magnitude from the different matrices. To indicatw the net mass transfer kinetics along treatments, the ratio of forward and backward rate constants ($k_1/k_2$) were used as a lipid-matrix partition coefficient. Results showed that in the PS matrix, all PAHs had a $k_1/k_2 < 1$ and with the increase of the PAHs hydrophobicity the $k_1/k_2$ increased, indicating a "hindering effect" from PS on the PAHs intestinal uptake in *D. magna*, and for PAHs with lower hydrophobicity, the "hindering effect" was higher. In the HA-PS and HA matrixes, all PAHs had $k_1/k_2 > 1$ and with increasing PAH hydrophobicity the value of $k_1/k_2$ decreased, indicating that the HA-PS and HA matrices enhanced the mass transfer of PAHs from the matrices to lipids, and for the PAHs with low hydrophobicity, the facilitation of mass transfer was more significant. Furthermore, anthracene was utilized to study the transfer efficiency. At the beginning of treatments, the transfer efficiency was larger than zero, and at 1.2 h and 1.0 h the efficiency decreased to less than zero in the HA and PS suspensions, which indicated a "carrier effect" of the matrix, implying that there were net mass transfers from matrices to lipids. In contrast, the transfer efficiency slowly decreased over time becoming less than zero at 5.0 h in the HA-PS suspension, which indicates that the intestinal uptake reached equilibrium after a period of time turning the matrix transfer into a "cleaning" process when the transfer efficiency was less than zero. The role of different uptake pathways to equilibrium bioaccumulation for treatments that contain different types of matrices were evaluated. Results showed that dermal uptake is the major route for the bioaccumulation of PAHs, while intestinal uptakes from singular or complex matrices at environmentally realistic concentrations are trivial. The effect of complex matrix on PAHs bioaccumulation was evaluated by a lipid normalized bioaccumulation factor (BAFL). Evaluations indicated that the bioaccumulation of PAHs in *D. magna* was through dermal uptake in the solution that had no complex matrix (i.e., no PS particles or HA). With increasing hydrophobicity of PAH, the logBAFL values substantially increased in all suspensions. Compared with the control groups, PS decreased the BAFL for anthracene, phenanthrene, acenaphthene and fluorene. For the highly hydrophobic PAHs, no significant effects were observed. The BAFL (of anthracene, phenanthrene, acenaphthene and fluorene) had no significant variation in both HA-PS and HA matrixes, whereas for pyrene and fluoranthene, an increase of 1.22–3.61 times and 1.75–2.78 times were observed respectively. The total concentrations of PAHs, as well as the affinity of NMs for *D. magna,* were decreased with the addition of HA.

Ma et al. identified the effects of co-exposure of PS NPs with phenanthrene (Phs) as a model PAH, as well as the bioaccumulation and environmental fate of $^{14}$C-Phs within freshwater systems using *D. magna* as a model organism [172]. They tested five different sizes of PS particles ranging from (50 nm to 10 µm). The effects of the PS particles on the bioaccumulation and transformation of Phs, which is mutagenic and carcinogenic to organisms, were determined using a radioactive tracer. Their findings revealed that the 50 nm PS at 10 mg $L^{-1}$ were toxic and caused significant physical damage to the thoracopods of *D. magna* when observed under a microscope, affecting their swimming and filter feeding behaviour by accumulating on the surface of the thoracopods. An additive effect was observed from the joint toxicity of 50 nm NPs and Phs when co-exposed to daphnids. For the bioaccumulation tests they performed a 14-day incubation experiment and showed that the presence of 10 µm MPs did not significantly affect the transformation, dissipation, and bioaccumulation of Phs while the 50-nm NPs showed a significant effect, enhancing the bioaccumulation of Phs-derived residues in the body of *D. magna*. This could be due to the higher adsorption capacity of Phs on the 50 nm PS NPs. The findings of this experiment confirm the importance of assessing both chemical and physical impacts and quantifying the bioaccumulation of the individual components and the mixture in order to have a better understanding of the interaction of NPs with hydrophobic pollutants in the environment.

Abdolahpur et al. investigated the role of dissolved organic matter (DOM) on the sorption of silver ions ($Ag^+$) onto 300 nm polyethylene (PE) and 600 and 300 nm polystyrene (PS) particles as models of nanoscale plastic debris (NPD) for 6 days [173]. *D. magna* was used as a model organism to determine how NPD affects the toxicity profile of $Ag^+$ in the absence and presence of DOM. Their findings demonstrated that under environmentally realistic conditions, the sorption of Ag ions onto NPD is influenced by the size and chemical composition of the particles. Their study showed that when using a constant particle number concentration for all treatments, a higher quantity of $Ag^+$ absorbed to the 600 nm PS-NPD compared to the 300 nm PS-NPD and PE-NPD. However, in the presence of 300 nm PS and PE, the toxicity of $Ag^+$ to *D. magna* was higher that when the 600 nm PS was present, implying that larger particles of NPD can be potentially less toxic than smaller particles of NPD even if smaller particles absorb a lower number of contaminants per particle. PE NMs sorbed a less amount of $Ag^+$ compared to PS of the same size (300 nm). However, in the presence of PE-NPD, the toxicity of Ag ions was higher in some cases, suggesting that the toxicity of NPD can be affected by the chemical composition of particles. Their findings also showed that, when DOM were present at concentrations of 1 mg $L^{-1}$ up to 50 mg $L^{-1}$, the sorption of $Ag^+$ onto the 600 nm PS have decreased, while the sorption of $Ag^+$ onto the 300 PE and PS increased when mixed with DOM. This study suggested that the Trojan horse effects of NPD may be inhibited by the presence of DOM in natural aquatic ecosystems. This study demonstrated the importance of understanding the relation between particle size, chemical composition and the role of DOM when evaluating the toxicity of NMs and determining the Trojan horse impact in model organisms.

## Potential role of chirality in nanomaterials mixture toxicity

Chiral substances possess a unique architecture such that, despite sharing identical molecular formulas, atom-to-atom linkages, and bonding distances, they cannot be superimposed [175]. In the environment and living systems, where specific structure-activity relationships are typically required for effect (on enzymes, receptors, transporters, and DNA), the physiochemical and biochemical properties of individual stereoisomers can differ significantly, and stereoselective metabolism of chiral compounds can influence pharmacokinetics, pharmacodynamics, and toxicity [175]. Environmental toxicology provides several examples in which bioaccumulation, persistence, and toxicity of molecules shows chiral dependence, and indeed many environmental pollutants are chiral, including organophosphorus compounds, organochlorines, pyrethroids, PCBs, polychlorinated dibenzo-*p*-dioxins and pharmaceutical contaminants [175]. Degradation of these compounds, as well as bioaccumulation, persistence, and toxicity of resulting metabolites often show chiral dependence.

Importantly, chirality is not limited to organic molecules, but also exists in inorganic compounds and crystals [176], whereby chiral compounds produce optically active crystals upon crystallization in which the spatial arrangement of the atoms are not superimposable with its mirror image [177]. There are many chiral crystals in nature with quartz, whose chirality arises from the helical arrangement of $SiO_4$ tetrahedra in the bulk structure, being the most common. CNTs also have intrinsic chirality, which arises from the manner the graphene layer is folded, as defined by a pair of integer indices (n, m) that denote the chiral angle θ (the degree of degree of helicity of the lattice) and the chiral vector (the roll-up direction) [178]. Chirality can be bestowed onto NMs by adsorption of chiral molecules [176], and by careful design of the crystal to expose chiral kinked and stepped surface structures - the kink sites lack symmetry being

'either left- or right-handed, and can be thought of as chiral, when the step lengths on either side of the kink site are unequal' [179].

NMs functionalised with different enantiomers (optical isomers) have been shown to exhibit enantioselectivity in interaction with receptors (for example) and different toxicities [176]. For example, surface chirality was shown to significantly influence the adsorption of blood plasma proteins (such as BSA and fibrinogen) onto $TiO_2$ films functionalised with L- and D- lysine or L- and D- tartaric acid [180], whereby the proteins had a stronger interaction with chiral centres on L-Lysine than on D-Lysine, resulting in more protein adsorption on L-Lysine and consequently more platelet immobilization and decreased platelet activation compared to the D-lysine surface. However, a mechanistic understanding of the effects of chirality on protein adsorption kinetics and thermodynamics (isotherms) is required in order to make predictions for other chiral functionalisation and for effects on NMs toxicity. CNTs with lower chirality were found to require shorter times than those with higher chirality to translocate through the membrane because of their weaker adhesion to the membrane [181]. A review of chirality of NMs provides further examples and readers are referred there for further details [175], where key recommendations include the need for development of three-dimensional structure-activity relationships, and consideration of local conditions, species and tissue differences and population polymorphisms which may additionally influence xenobiotic effects of chiral compounds and chiral NMs [175].

From a mixture toxicity viewpoint, chirality needs to be considered in terms of the NMs' intrinsic chirality, as well as the potential for enantiomer-specific or preferential surface functionalisation and specificity of subsequently protein binding and cellular adhesion as a result of intentional or self-selective enantiospecific ligand enrichment at the nanomaterials surface. To date, chirality has not been considered much in nanosafety or mixture assessment, and no data has been found specifically associated with impact of chirality of NMs on their toxicity to *D. magna*. However, it is known that daphnids are differentially sensitive to enantiomers of pesticides resulting in an order of magnitude difference in acute toxicities of the individual enantiomers and the racemic mixture, which has implications for ecological risk assessment for chiral entities [182].

## Nanoinformatics approaches: From computational simulation to data-driven science

Given the early stage of development of NMs mixture toxicity, it is clear that predictive models of mixture toxicity have not yet even begun to consider the additional complexity introduced by NMs with their enormous surface area for co-pollutant binding and thus their potential as carriers for other pollutants, as described by the Trojan horse conceptualization [4,52–55,52]. However, very significant progress has been made in terms of the prediction of toxicity of NMs themselves, including prediction of their biomolecule (protein and small molecule) coronas and their cellular adhesion, uptake and toxicity, which form a strong basis upon which to develop NMs mixture toxicity models. This section provides an overview of the progress to date and the current state of the art, considering both physics-based materials models and data-driven toxicity-focussed models, and provides recommendations on where rapid progress could be made towards implementation of NMs mixture toxicity models.

The ecotoxicity evaluation of pollutant mixtures presents a great challenge due to the lack of standard prediction models coupled with the lack of ecotoxicological data for many of the individual compounds. The release of NMs into environmental systems adds to the complexity of this problem [1–3]. Considering the enormous variety of NMs properties and their high potential to interact with environmental compounds (e.g. organic matter, biomolecules, metals, organic pollutants) and biological systems, multidisciplinary strategies are essential to the proper risk evaluation of these materials [183]. Computational approaches have already begun to provide valuable advances in the understanding of mechanistic aspects of nano-bio interactions, and in the development of predictive models employing data-driven approaches [184,185], which made them increasingly important in mixture toxicity assessment and regulation. Correlation between NMs properties and their biological/environmental effects have thus been extensively pursued in the field of nanotoxicology [183,186]. While great advances have been achieved in this area, they also led to increasingly complex questions, which seek to comprehend the role of environmental components in NMs toxicity and vice versa [15]. Once in biological environments, NMs behaviour is complex and involves a series of phenomena occurring at different scales, which comprise interactions with molecules, cells, and organisms, as well as changes in NMs structure and dispersion [187]. Therefore, to comprehend the mechanisms involved in the destination of these materials in biological medium, it is necessary to outline strategies that access and correlate across these scales [188]. In this context, the application *of in silico* approaches is advantageous to understand phenomena that cannot be easily accessed experimentally and to evaluate the primary NMs properties responsible for them [27,30,187]. Current physics-based simulation approaches can reveal phenomena at molecular, atomic, and electronic detail in systems with different level of complexity; well-known methodologies include coarse-grained (CG), molecular dynamics (MD) and Density Functional Theory (DFT) models [189–193]. These techniques are useful to describe the structure and physicochemical properties of NMs, to evaluate their interactions with molecules, including protein binding and cellular attachment, and to elucidate the surface characteristics that promote NMs' agglomeration, dissolution, oxidation, catalytic activity among other reactions (Fig. 8) [183,189,194].

In view of these dynamic and evolving interactions of NMs with their surroundings, surface adsorption studies can provide important insights about the toxicity mechanisms of pollutants and NMs in co-exposure scenarios. The characteristics of these interactions (e.g., adsorption energy, chemical sites involved etc.) have an important role in the fate of NMs and co-pollutants in organisms and the environment, and consequently, in the hazards arising from mixtures [187,197–199]. Geitner et al. showed that the presence of sites specific to different types of interactions favours the adsorption of pesticides to NMs surfaces and impacts the environmental fate of the adsorbed chemicals [200]. They selected a library of 15 pesticides, and 4 different NMs of natural or incidental sources (i.e., $C_{60}$ fullerene, fullerols with 8 (fullerol-8) and 24 (fullerol-24) hydroxyl groups, and ceria NMs) to evaluate interactions of all possible pairs through MD simulations. Due to the presence of sites for different types of adsorption mechanisms, such as hydrogen bonding, van der Waals, and π–π interactions, fullerol-8 NMs presented the strongest interaction with most of the chemicals tested. Furthermore, experimental adsorption assays showed that most pesticides preferentially interacted with NMs rather than clay, which emphasized the role of NMs in the fate of co-contaminants and the necessity of further studies that explore the complexities of NMs behaviour in environmental media.

Similarly, when used to evaluate reactions in which NMs may be involved in the environment, modelling approaches may provide valuable information about catalysis, aging and degradation processes involving these materials [201,202]. Moreover, NMs which present different catalytic properties may elicit reactions involving any coexisting pollutants and generate more toxic products, even when the NMs are used for environmental remediation purposes. Detailed analysis of the reactions pathways and kinetics, including with co-pollutants other than their intended targets, is essential to ensure the environmental safety of these materials and can be performed by ab initio methods [187,203]. For example, Wei et al.

utilized DFT to evaluate and compare the mechanisms, kinetics and by-products of the photocatalytic degradation of 4-chloroguaiacol (4-CG) catalyzed by graphene, boron-nitrite (BN), and carbon-doped BN (BCN) nanosheets [204]. The authors showed that BCN nanosheets are a promising material for catalysis, due to its excellent adsorption capacity for 4-CG, band gap, which narrowed from 3.81 to 1.54 eV by C-doping, and its suitability for visible light catalysis. Furthermore, BCN nanosheets changed the catalysis reaction mechanism compared with other NMs, reducing free energy barriers and increasing degradation rates. Toxicity analysis of the degradation products showed that all transformation products were harmful to at least one organism tested (i.e., fish, green algae, or daphnia), drawing attention to the need for evaluation of potentially toxic products that can be formed during pollutant degradation. The potential impact of chiral functional groups on NMs surfaces on effectiveness of degradation, and indeed on differences in rates of environmental degradation of co-pollutant enantiomers may also play an important role, which has not yet been considered in the experimental approaches nor in modelling.

Despite simulation methodologies enabling deeper understanding of the processes of bio-nano interactions and giving important insights about the mode of action of NMs and pollutants (eco)toxicity, they have significant limitations currently. They require extensive expertise and computational knowledge, and their high computational cost limits model complexity, which made these approaches inadequate for the prediction of toxicity end points with complex mechanisms (e.g., genotoxicity, reproductive toxicity, apoptosis, etc.).[27,30,205] Therefore, in order to develop predictive capacity for the (eco)toxicity of NMs and pollutants in environmentally realistic media, statistical and machine learning (ML) methodologies, which are capable of dealing with such complexity, are required. These methods utilize algorithms to assess a great volume of data and can model the relationships between significant descriptors (e.g., structural and molecular properties of NMs and chemicals) and phenomena of interest (e.g., biological endpoints) obtained experimentally or theoretically [206,207]. In this context, physics based modelling methodologies are also applied to provide descriptors that can be hard to measure and are useful for predictive analysis, which include intrinsic NMs properties, such as consistent or electronic properties (e.g., HOMO-LUMO gap, enthalpy of formation, molecular weight, cation charge, metal electronegativity), and extrinsic NMs properties, which are dictated by the surrounding medium (e.g., hydration energy, contact angle for water, dissolution rate, surface charge density and more) [208–210].

Data driven approaches (e.g., machine learning, Quantitative Structure-Activity Relationships (QSAR), deep learning) represent a new paradigm in science, in which new knowledge is extracted from data by identifying patterns and correlations that are not easily detected in individual results or are impossible to visually analyse due to the large amounts of complex and multidimensional data

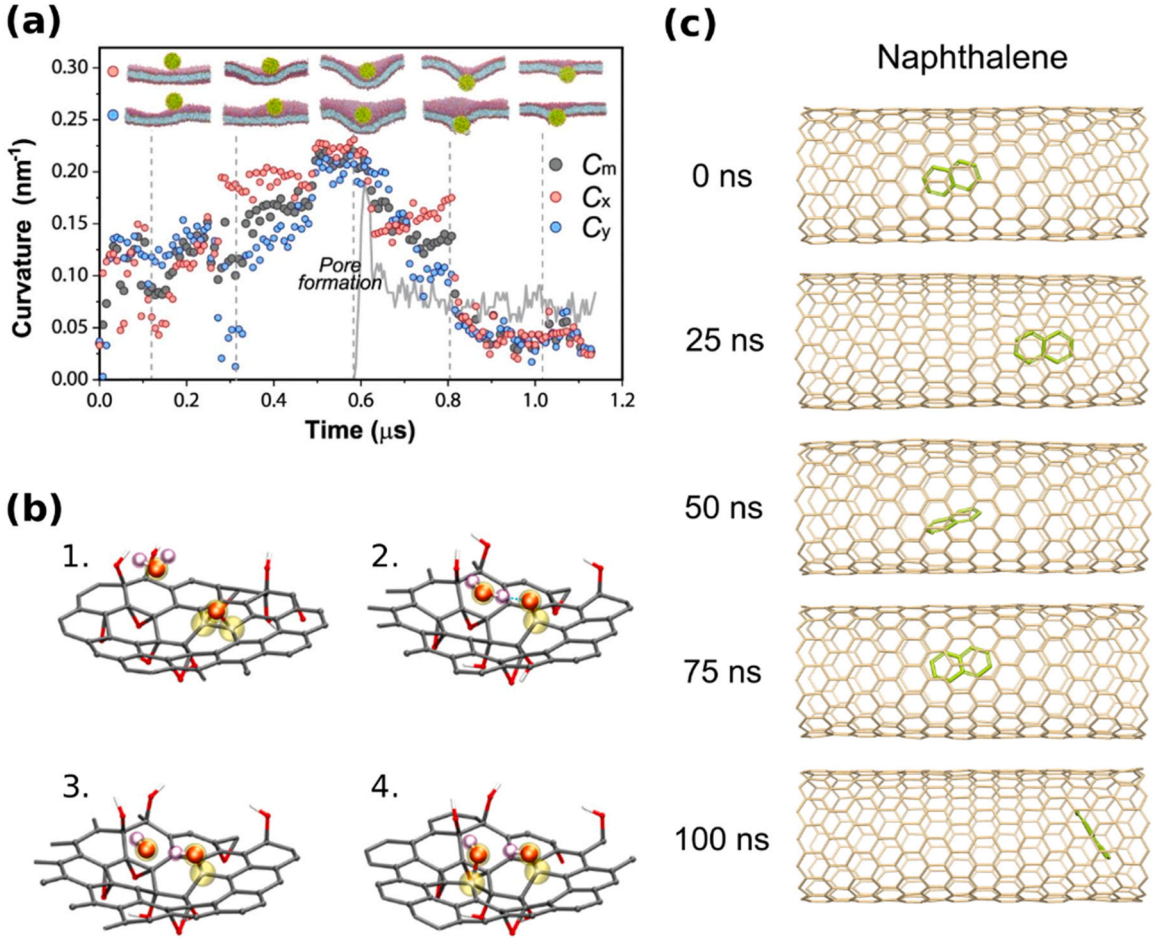


**Fig. 8.** Examples of application of different computational simulation approaches to understand the interface between NMs and the biological environment. a) Coarse-grained simulation of the translocation of NMs across model cell membranes. Analysis of membrane curvature vs. simulation time. Reproduced from ref. [195] Copyright 2020 Springer Nature. b) *Ab initio* study of reactive event chain in the GO interface with water. 1- Epoxide and water. 2- Epoxide opening to form an alkoxy and a carbocation. 3- Water hydrogen abstraction by the alkoxy to form a hydroxy group. 4- Addition of the hydroxide onto the carbocation to form a new hydroxy group. Reproduced from ref. [194] Copyright 2020 American Chemical Society https://pubs.acs.org/doi/10.1021/acs.jpcb.0c05282 c) Molecular dynamics simulation of binding of naphthalene to a carbon nanotube. Reproduced from ref. [196] Copyright 2020 Elsevier.

[206,211]. A great volume of data have been generated over decades, and the advances in experimental and computational techniques (e.g. High-Throughput approaches) have resulted in even greater amounts of complex data, making necessary the development of new methods to analyse and visualize it, in order to optimize the extraction of knowledge from the data [206]. In biological science, for example, associated with the advance of molecular biology and genomics, bioinformatics has been demonstrating successful application of data science in the last decades [212]. Currently, there are numerous databases with vast information regarding DNA and RNA sequences, protein structures and functionalities, metabolites, among others, that are fundamental to understanding biological processes at different levels of organization [213]. Taking this as inspiration, in materials science, the Materials Genome Initiative was created with the purpose of overcoming the trial-and-error research and optimizing the development of advanced materials by supporting a rational integration of theoretical (e.g., computational methods) and experimental approaches, and the building of collaborative databases [214–216]. Several databases filled with structures and properties of materials, experimentally or theoretically calculated, have been used in the discovery of promising materials for diverse applications by the combination of data-driven methods, computational simulation, and experimental testing in an optimized workflow [217,218].

Since the advent of nanotechnology, many different NMs have been synthesized and tested for a wide range of applications. In this period, it became clear that NMs' properties (e.g., electronic, optical, mechanical properties), as well as the nano-bio interactions and the biological/environmental effects of these materials, are strongly related to their characteristics such as composition, size, shape, and surface chemistry. [186,219]. However, it is still a challenge to track these correlations and delineate models to predict the properties and biological behaviours of NMs at the design stage. The obstacles faced in this task include the lack of standardization in methodologies, the intrinsic structural variability of the samples (e.g., particle size distribution, heterogeneity in shape and degree of agglomeration), poor sample characterization (e.g., the potential for / impact of NMs chirality is rarely considered), and a limited number of systematic studies of NMs' interactions in biological environments [220–222]. Therefore, in order to advance to a rational development of NMs following the safe-by-design concept, multidisciplinary and collaborative approaches, and extensive dialogue between experimental and computational researchers to design experiments that maximize the available data and thus the modelling potential, have been shown to be indispensable [223].

Great efforts have been made in the field of nanotoxicology towards the integration of computational and experimental methodologies, as well as the organization and storage of data in common databases to enable the application of machine-learning analysis [27,185]. To facilitate this, projects such as the Horizon2020-funded NanoCommons and NanoSolveIT are creating the infrastructure necessary for computational modelling, data sharing, and tool development, as well as establishing standard experimental and modelling procedures, ontologies, and reporting templates. These methodologies and tools, developed to collect, organize, validate, store, share, model, analyse nanosafety data (termed nanoinformatics), are intended for application in decision-making and regulation of nanotechnologies [27,30,205].

For data-driven modelling, the general concept of data consists of an aggregation of numerical and textual information in the format of matrix (Fig. 9), in which each input (row) is described by a number of descriptors or features (columns) and represent the object of study that can be, for example, a NM, a sample, an image, etc. The data may also present defined target properties (also called labels), which consist of specific properties to be predicted, whether numerical or categorical, for example, toxicity endpoints (e.g., cytotoxicity, bioaccumulation, lethal dosage). In this case, the matrix of inputs X is related to a vector Y containing outputs (indicated as (Y) in Fig. 9). Depending on whether the model's target properties are available or not, different types of machine-learning algorithms are applied [30,206].

Unsupervised techniques are used for data that has not been harmonized or curated. This type of algorithm utilizes statistical analysis for grouping of data based on similarity, which may be used to elucidate hidden patterns and relations in the dataset when there is no type of classification or categorization, or to reduce the dimensionality of the data to decrease computational cost and complexity in processing information. Examples of unsupervised algorithms include K-means, Hierarchical Cluster Analysis, Principal Component Analysis, and Density-Based Spatial Clustering [30,222].

On the other hand, supervised algorithms are used when there is a target variable or property defined in the data set. These techniques apply mathematical and statistical strategies to find the function that lead the input data to the target (output) [206]. Supervised methods are able to predict either numerical (e.g., No Observed Effect Level - NOEL, lethal dosage) or nominal (e.g. toxic, non-toxic) outputs, which are characterized as regression or classification problems, respectively [209]. The predictive capacity of these methods makes them especially interesting for the ecotoxicology context, in which the outputs consist of biological endpoints, such as lethality, bioaccumulation, reproduction effects etc [45]. In that way, as hazard and exposure data are obtained, the algorithms can be used to fill missing values or classifications for non-tested conditions in a faster and more cost-effective manner than via experimental testing [220,222].

Currently in nanotoxicology, predictive models are mainly based on NMs structural features, and the main approach used for that is Quantitative nanostructure–activity relationships (QNAR). In this approach, the predictions are based on the assumption that NMs with similar properties present similar biological effects [224]. Machine-learning algorithms such as regressions [225], K-Nearest Neighbours [226], support vector machine [227], artificial neural network [228], decision tree [229], among others, play an important role in development of these models. Depending on the algorithm applied, the correlations found by the models are interpretable and can give important insights for deriving causal relationships, however QNAR models normally do not provide direct mechanistic interpretations [209].

In order to develop accurate and reliable predictive models (for NMs alone or for environmental mixtures), the first task is to have a good definition of the problem which needs to be solved, in order to be able to properly proceed in the machine-learning work-flow, as listed below and illustrated in Fig. 10 [206,230].

(1) *Dataset Formation*: Data collection from existing literature and databases or from new experimental results, and extraction of the information related to the defined problem, features, and endpoints. Organisation of the data into a matrix (as shown in Fig. 9) with one row for each NM / co-pollutant combination at a specific concentration and timepoint.
(2) *Data Processing*: This step involves cleaning of data by removing corrupt or incomplete data. Feature selection and reduction is then applied, to reduce the irrelevant and redundant information by identifying those descriptors that are most predictive / explain most of the variability in the dataset. Transformation of data by normalization, discretization, averaging, etc. is then applied, and finally a balancing of the representativeness of the outputs categories is performed to ensure a reasonable distribution of (for example) toxic and non-toxic outcomes in the training set.
(3) *Data representation and transformation*: This involves formatting of data into an understandable presentation for algorithms, for

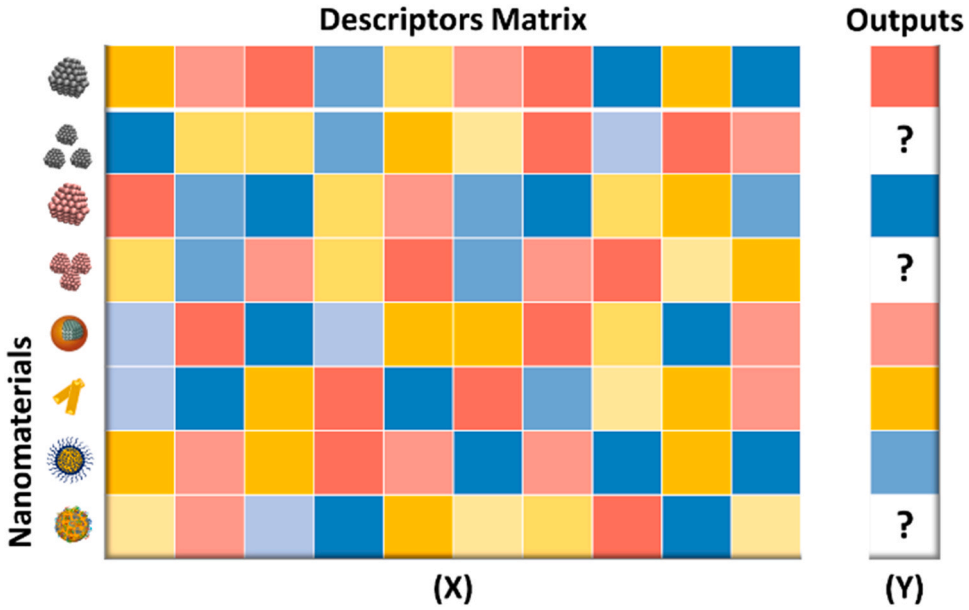


**Fig. 9.** Data matrix for machine-learning applications. For nanoinformatics, the matrix (X) contains the descriptors (columns) that represent individual NMs (or their mixtures with co-pollutants) (rows). This matrix may be related to specific information (Y) regarding the impacts of the NMs or their mixtures, for example, toxicity endpoints. Machine-learning algorithms are then trained with the known data to predict the missing values (?) in Y, and thus can predict the behaviour of NMs for which limited data is available within the limitations of the domain of applicability which is determined by the properties of the training set of NMs.

example, representation of nominal features in quantitative formats, representation of the object of study, such as a NM, by a set of relevant features, etc.

(4) *Machine Learning algorithm application*: The algorithm must be chosen and trained according to the defined problem. It is essential to pay attention to the algorithm's characteristics such as accuracy, performance, training time, quantity of data required, complexity and interpretability of results. The dataset is divided into training, validation, and test sets.

(5) *Model validation and optimization*: The initial analysis of the model prediction efficiency, and optimization of the models' hyperparameters is executed utilizing the validation dataset. When an optimal set of parameters is reached, the performance and accuracy of the model is evaluated using the test dataset, this step reveals if there are inconsistencies such as bias, variance, or under/overfitting. If the model failed in prediction and accuracy, the previous steps are repeated, since data selection should be improved, or the learning algorithm should be changed.

(6) *Application*: The validated model is applied in prediction or classification of unknown data and may be further improved or its domain of applicability extended as more data become available.

To date, several studies have been published presenting computational models to predict NMs' properties and biological responses, and for use to develop new NMs through safe-by-design approaches [210,225,228,231–236]. For example, Le et al. developed predictive computational models to relate NMs' properties to cellular impact analysing experimental data of NMs characterization (i.e., size, aspect ratio, doping type, doping concentration, and surface coating) and toxicity endpoints (i.e., cell viability, membrane integrity, and oxidative stress) of a library of 45 ZnO NMs. They found that the NM concentration the cells are exposed to, the type of surface coating, the nature and extent of doping, and the aspect ratio of the NMs are all important factors in the cellular toxicity of the NMs tested [225]. On the other hand, by implementing an electronic structure-based descriptor, Shin et al. were able to calculate similarity and predict cytotoxicity and zeta potential of ZnO NMs with different concentrations of dopants (i.e., Fe and Co). The new descriptor, called a size-dependent electron configuration fingerprint, was capable of describing the complex structure and composition of doped NMs in a simplified manner [231]. Given the generally poorer understanding, as yet, of the role of chirality in NMs toxicity it is not that surprising that nanoinformatics models have yet to consider chirality either intrinsic to the NMs or as a result of functionalisation of the NMs with stereoisomers. However, the emerging evidence of enantiomer-specific protein binding and cellular attachment and uptake, and the prevalence of chirality in environmental co-pollutants, provide clear imperative for inclusion of chirality in NMs mixture modelling and will be an important feature to address in mixture toxicity. That said, several machine learning models have been developed to predict the chirality of small molecules and their assemblies into metamaterials [237]. Additionally, machine learning approaches such as deep feedforward neural networks and graph neural networks for conformal prediction (of enantiomers) have been developed and evaluated in terms of their performance on data from the Tox21 challenge, indicated that the resulting models were highly predictive with high confidence levels [238].

Despite the advances, there are many challenges to be overcome in order to develop reliable mixture toxicity models for NMs, as well as to completely comprehend and modulate the NM's characteristics involved in (eco)toxicity. One important issue comprises the availability, quality, organization, and standardization of data. Normally, the available high-quality datasets of NMs biological assays are small and lack complex biological response information (e.g., Adverse Outcomes Pathways (AOP)), which limits the applicability domain of models developed from these datasets. Another challenge is the complexity inherent to accurately represent different NMs by useful descriptors for machine-learning algorithms [239]. These descriptors include: NMs characteristics (e.g. surface coatings, composition, shapes, crystallinity, size distribution), their intrinsic properties (e.g., electronic structure, reactivity, ionization energy, electronegativity), as well as their environmentally modulated properties (e.g. biomolecular coronas, superficial charges, agglomeration, loss of coating, dissolution rates etc.) [214,223,231,240]. Furthermore, in mixture studies the implications of all these NMs properties and the environmental transformations of the NMs have to be considered in

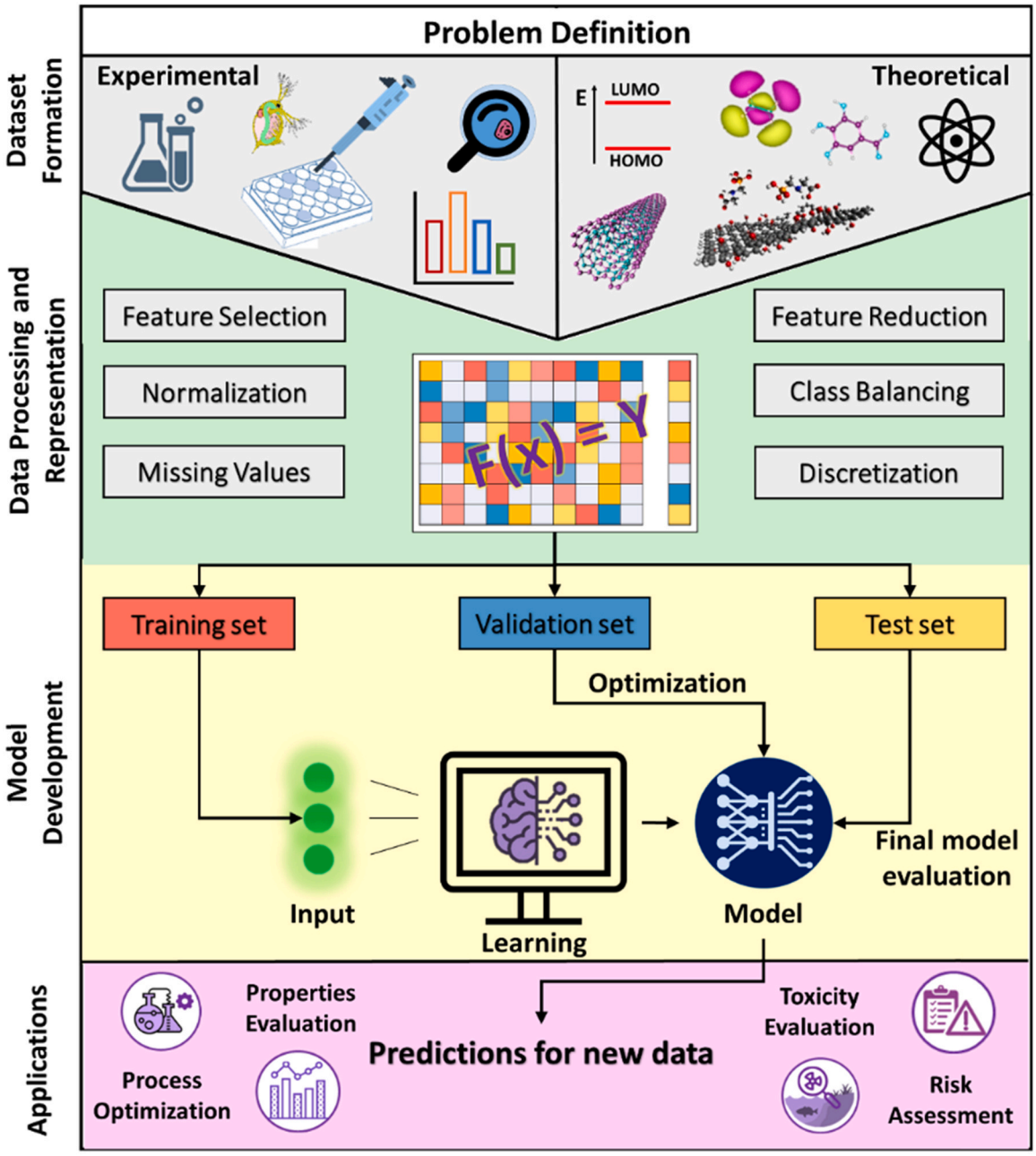


**Fig. 10.** Supervised hypothetical machine-learning workflow for applications with combined toxicity of nanomaterials and environmental pollutants.

terms of their influence on adsorption affinities and binding constants of co-pollutants.

Diverse computational strategies can be used in overcoming these challenges. For instance, when limited data is available, different predictive approaches are used for filling data-gaps, a common methodology for which is read-across [44,222,241,242]. In this method, target properties or biological endpoints of NMs are estimated from the data available for structurally similar materials, previously grouped by unsupervised machine learning techniques [243]. Besides that, computational algorithms are important in the generation of technologies that accelerate and increase the accuracy of obtaining experimental data. Recently, Karatzas et al. developed a deep learning-based (i.e., Neural Network-based) algorithm to automatically detect and classify the adverse effects of different NMs to *D. magna* by analysing microscopy images generated from reproductive assays over multiple generations with continuous versus parent (F0 generation) only exposure. The algorithm was trained with over 4000 light microscopy images of *D. magna* exposed to different NMs and in various conditions, including different media, and freshly dispersed versus environmentally aged NMs. At the end, the model was capable of separating the regions of the organisms (i.e., head, eye, tail, abdomen, heart, etc.) potentially affected by the NMs and could classify the level of injury caused by different NMs [45].

As the understanding and prediction capacity of NMs toxicity advances it becomes clear that the environment characteristics and its components are determinant in NMs' interactions with biological entities. In this context, mixture toxicity has been increasingly discussed in the nanotoxicology and nanoinformatics field. The predictability of joint effects of NMs and other components of the environment (i.e., NM-mixtures) is of great importance for environmental risk assessment, however, these studies are still in their infancy. The requirements of quality data, development of new descriptors and models are even more demanding than for single

toxicity models. Therefore, standardized, and reliable methods for estimation of NM-mixture toxicity are still lacking. According to Zhang et al. an initial strategy is to incorporate the common methods applied to chemicals mixture toxicity, such as independent action and concentration addition [244]. There are already examples in the literature of application of descriptors based on chemical mixtures to development of models for heterogeneous materials [210,233,236] and joint toxicity of soluble NMs and their dissolution products [133]. Furthermore, another important aspect is utilizing computational models to accelerate the process of obtaining the required input data. Advances in that direction include predictive models for molecular adsorption onto NMs surface, either for pollutants [245,246] or biomolecules [247–249], as well as exploration of omics approaches [228,250,251] which are essential for mechanistic understanding of NMs mixture toxicity. Indeed, a recent publication has indicated that even in the absence of dose-response data, a random-forest QSAR model was developed that was more predictive than either the CA or IA models for prediction of the immobilization of daphnids by mixtures of $TiO_2$ NMs and selected co-pollutants [62].

Computational chemistry methods such as QSAR, which are already successfully applied in the field of toxicology of chemicals, have been gaining attention for regulatory purposes by government agencies [230,252,253]. The REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation created a list of conditions that must be satisfied in order to justify the application of (Q)SARs instead of experimental analysis, including specificizing the validation and documentation requirements, requiring utilization of models according to their applicability domain, and ensuring that the results are adequate to the needs of risk assessment [254]. In addition, according to the Organisation for Economic Co-Operation and Development (OECD), to be suitable for regulatory purposes, (Q)SAR models should present a well-defined endpoint, an unambiguous algorithm, a defined domain of applicability, appropriate measures of goodness-of-fit, robustness and predictability, and, when possible, a mechanistic interpretation of the mode of action of the toxicants on which the model is based [255]. These principles are fundamental, and extendable to any *in silico* modelling approach.

The relevance of understanding NM's biological/environmental behaviour for proper risk assessment and regulation of new technologies is undeniable. It is clear that computational techniques are valuable to access complex phenomena, either by analysing specific pieces of the important processes, such as interactions of nano-bio interface, or correlating complex data sets to biological endpoints [187,211,256]. However, there is no absolute approach capable of dealing with all aspects of intricate systems, such as biological and environmental media. In this context, recent experiences from bioinformatics and the Materials Genome Initiative have shown that the integration of computational and experimental methodologies by the cooperative interchange of information provides a powerful strategy to advance towards complex questions such as NMs mixture toxicity [213,215]. The combination of physics-based simulation with advanced ML & AI techniques will lead to the development of advanced chemo-/nano-informatics models [257]. Molecular docking or/and molecular dynamics methodologies can be applied to protein systems to recognize proper ligand placement that facilitates complex stabilization. While ligand-based models (mL/AI) can provide an initial estimation of the toxicity of compounds, they often result in low specificity. The combination of the two computational techniques in a consensus manner that utilizes multiple sources of information rather than just structural or ligand assay data improves the specificity of the procedure. Another promising approach is to incorporate the complexity of nanomaterial and co-pollutant features, alongside their environmental conditions, is the emerging meta ensemble approach, which is a deep learning framework for quantitative toxicity prediction that integrates the outputs of a set of models for individual endpoints (of which chirality impacts on protein corona and/or cellular attachment could be one) into an overarching toxicity prediction. This meal-model deep learning approach has recently been demonstrated by Karim et al., using five individual deep learning models each with their own base feature representations (including physicochemical descriptors, low-level fingerprints, SMILES etc.), which are then integrated using a separate deep learning model to perform aggregation of the outputs of the individual deep learning models [258]. The demonstrated version, for small molecule toxicity, trained the deep learning models by combining four quantitative toxicity data sets including: an $LD_{50}$ dataset indicating the lethal dose data for killing 50% rat population when a given compound is administered orally; an $IGC_{50}$ dataset which shows the concentration of a chemical compound needed to arrest the growth of *Tetrahymena pyriformis* when exposed for 40 h; an $LC_{50}$ dataset on fathead minnow, a species of temperate freshwater fish, after 96 h exposure; and an $LC_{50}$-DM dataset which indicated the concentration of a compound in water (mg/L) causing 50% population of *D. magna* to die after 48 h. The outputs of the base learning models were used as the meta features for the meta-learning model. High-level physicochemical, low-level fingerprints, SMILES-embedded vectors, and fingerprint-embedded vectors when used to create meta features for the meta ensemble model to enhance the performance over a wide range of metrics for the quantitative toxicity prediction tasks [258]. Thus, a promising direction for NMs mixture modelling could be to integrate the best, and most relevant existing NMs models, including for binding kinetics and competitive adsorption to NMs surfaces, and those for co-pollutant and /or mixture toxicity into meta models utilizing knowledge graphs, neural networks and other deep learning approaches.

## Regulatory issues

Regulation of nanotechnologies is currently under discussion worldwide towards promoting safer and sustainable utilization of NMs. As highlighted in this review, *Daphnia* is a key aquatic organism for ecotoxicity assessment of chemicals and NM and is an ideal species for computational approaches including deep learning. The OECD recommends a defined synthetic medium for the harmonized testing of chemicals in acute and chronic ecotoxicity testing using the *D. magna* immobilization and reproduction tests [259,260], since the goal of standard testing is to facilitate comparison of chemicals under identical conditions for ranking of toxicity, rather than to provide environmental realism. While this is ideal for soluble chemicals, these tests are not optimized for NMs with properties that can be determined by their environmental surroundings, a feature referred to as having extrinsic properties as well as intrinsic ones [127,261,262], leading to suggestions for modification of the test guidelines to include natural organic matter (NOM) or utilization of daphnia conditioned medium [56,89].

The highly reactive surfaces of NMs means they can interact with themselves or surrounding biomolecules and chemicals, suggesting that the lack of dispersing agent in the OECD Daphnia tests compromises the ability of the tests to rank NMs toxicity. Chronic transgenerational studies in the presence and absence of environmentally relevant media (with differing NOM contents), with the use of transformed NMs that are more representative of environmental pollutants, have demonstrated that the standardized Daphnia tests currently overestimate NM toxicity to the first exposed generation yet fail to consider potentially more severe impacts in subsequent generations [56]. These results demonstrate the importance of updating standard testing to reflect scientific advances and increase stakeholder trust in regulation. However, for NMs, the ranking will be meaningless if incorrect forms are assessed compared to those present in the environment. Similarly, for assessment and ranking of mixture toxicity, if mixtures including NMs are

evaluated in the absence of competitive interactions arising from biomolecules and without considering the impact of biomolecules on the bioavailability and retention of co-pollutants at the NM surface, the results will be meaningless [89].

Regulation of complex mixtures is increasingly important as most regulatory guidelines available nowadays are focused on single chemicals or commercial formulations (combinations of well-known chemicals) [263,264]. Evaluating and predicting toxicity of individual small molecules or nanomaterial in the environment does not provide realistic information as in the majority of the cases exposure occurs as a mixture rather than to a single component, and thus organism responses are to the multiple challenges simultaneously. Thus, evaluation of single component toxicity for specific species and environmental compartments may be misleading and may underestimate aspects such as overload or threshold exceedance through additive effects. However, the assessment of a mixture's toxicity is much more complex than toxicity evaluation of a single component material or chemical, as the interactions among the individual components of a mixture can significantly change the apparent properties of its components. For instance, the components in a mixture can present additive behaviour of response/effects or may induce either increased (synergistic) or decreased (antagonistic) effects [13]. A recent review by Kar & Leszczynski summarized the advantages of chemo-/nano-informatics methodologies for the prediction of the toxicity of mixtures and multicomponent materials, including the fact that: (i) the *in silico* methods can be applied for the replacement of animal testing for toxicity purposes; (ii) the developed chemo-/nano-informatics approaches can be applied for the prediction of unknown mixture combinations [13]. This is especially important for the majority of mixtures since toxicity data are missing for the individual components and thus predictive models can be used to fill the data gaps also. Since a single model cannot be applicable for the whole universe of chemicals and materials, the domain of applicability determination it is a crucial step for the identification of the area of the reliable predictions. Several regulatory agencies (US EPA, ECHA, EFSA, Health Canada) have already applied chemo-/nano-informatics predictive models for the toxicity and risk assessment of chemicals and NMs. Recently, with the development of advanced tools based on mL & AI, chemical and NMs mixture risks can be quantified with high reliability in a cost and time effective manner compared to the experimental methods (in vitro and in vivo).

Methodologies for assessing risks from combined exposure to multiple chemicals have been developed for different regulatory sectors, however, a harmonized approach for evaluating combined exposure and management across different regulatory sectors is lacking [263]. There is no consensus currently by the legal authorities of US, Europe, Canada, and Brazil about regulating mixtures of chemicals in our changing environment, especially, considering real and dynamic exposure conditions, despite this being an increasing concern [1,2,265]. In fact, there is a general perception that testing of chemicals on an individual basis does not reflect real conditions in the environment, where organisms are typically exposed to various chemicals at the same time. Indeed, the OECD recently published a document with considerations for risk assessment of combined exposure of multiple chemicals [7]. Furthermore, additional attention will be necessary for NMs in mixtures/combined exposures considering the unique properties of these innovative nanoscale materials, including competitive binding, ageing, dynamic transformations, dissolution, etc. Within the EU, REACH has was updated in 2018 (Regulation 2018/1881) to require information on NMs to be reported by January 2020, focussing on nanoforms, which consider changes in size, shape, coating etc. for a specific composition, and sets of nanoforms which despite slight variations in their physico-chemical properties are demonstrated to behave similarly from a toxicological perspective [266]. However, this does not consider potential differences in biomolecule interactions, nor in adsorption of co-pollutants as yet, but as functional assays for these end-points become available they could easily be incorporated into the evidence basis required to demonstrate similarity. To the best our knowledge, there is no specific guidelines available for assessing the effects of NMs in mixtures using validated and harmonized protocols at moment. For NMs containing more than one substance, their toxicity has to be evaluated or predicted in order to fulfil the requirements of CLP regulation (Classification, Labelling and Packaging of Substances and Mixtures), which implements the United Nations's Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) [44,267] Ultimately, the Water Framework Directive, Green Deal and Circular Economy policies and regulations may provide a stronger driver for adoption of mixture toxicity, with a focus on reduction of undesirable emissions into the environment in the first place. In the near-term, the development of Integrated Approach to Testing and Assessment (IATA), implemented by state-of-the art computational methods, underpinned by an expert decision-support system that minimizes the time and cost required for NMs mixture risk assessment is the most likely approach.

## Conclusions and perspectives

Based on the studies reported so far, there is still a long way to go to fully comprehend the toxicological effects of chemical mixtures containing NMs in the environment. The lack of standardized assays that account for the dynamic reactivity of NMs, consistent NMs characterization, and colloidal stability studies makes it difficult to compare results and understand the properties and mechanisms behind the experimental results reported in the literature or NMs environmental mixture toxicity, even focussing on just one species, as we did here using *Daphnia* as the model organism. There are several environmental parameters (e.g., pH, ionic strength, organic matter, biomolecule content, NMs ageing and transformation) that must be considered when addressing mixtures toxicity with NMs, due to their strong influence on the ecotoxicological outcomes. A fundamental gap noted in the literature on NMs mixture studies to date has been the lack of competitive binding studies in co-exposition scenarios, such that all NMs potential binding sites are accessible to the co-pollutant, increasing its uptake/bioavailability, whereas, in reality, many of the binding sites would be occupied by NOM or other molecules. Indeed, molecular interactions of the co-pollutant with NOM might also influence its subsequent release from the NMs surface following ingestion by organisms. Similarly, the impact of chirality, both intrinsic to NMs but also that arising from surface functionalisation of NMs and that inherent in many co-environmental pollutants on binding to the NMs surface and attachment to cells and organisms is an important, as yet overlooked, factor that needs to be considered in both the assessment and prediction of NMs mixture toxicity. Thus, a clear recommendation from this paper is that NMs mixture studies utilize conditioned medium or add biomolecules at environmentally realistic concentrations when assessing combined (and indeed individual) toxicity.

The potential for nanoinformatics and computational approaches in NMs-mixtures toxicity is enormous; nanoinformatics will bring great benefit to this area, establishing new standards, harmonized experimental protocols, ontological terms and common language, as well as enhancing storage and sharing of data, which will help to optimize the availability of information, either experimental and computational, by applications of the FAIR principles and data-driven application for nano-ecotoxicity modelling of complex mixtures in the environment. Integration of existing modelling approaches for chemicals mixtures with recent advances in simulation and machine learning applied to NMs toxicity will lead to rapid progress. To achieve NMs mixture toxicity assessment and prediction, we highlight here some important areas for future research and development:

- Physico-chemical characterization of NMs is vital, especially, the surface chemistry properties of NMs and their influence on binding of organic and inorganic contaminants. Predictive models are emerging for this, but a strong focus on competitive binding scenarios and evolution of the corona composition as the NMs move through the environment and through organisms is needed.
- It is critical to develop harmonized protocols for performing co-exposure toxicity testing with nanomaterials, in order to determine if the effects are synergistic or agonistic, and to explore the on/off kinetics of the co-pollutants relative to the uptake and biodistribution of the NMs in organisms.
- The impacts of the eco-corona formation on NMs and binding of co-pollutants needs investigation during mixture toxicity experiments. Harmonized protocols for conditioning media, corona characterization and competitive binding assays are critical aspects to be considered.
- Analytical methods to confirm both NM and co-pollutant accumulation and tissue distribution are required, and models for the transport and release of co-pollutants from NMs coronas under different physiological conditions would enable enhanced understanding of where cargos are released (e.g., in the organism gut or only in lysosomes following internalization) and at what rates, and how this is affected by various NMs transformations (agglomeration, dissolution, sulfidation, enzymatic biodegradation etc.).
- Adverse Outcomes Pathways (AOPs) are a promising approach in (eco)toxicology, especially, for understanding the impacts of low-dose mixtures at environmental realistic exposure conditions. While good progress is being made for chemicals, NMs introduce a range of challenges, for all the reasons described above, meaning that additional effort is needed to establish them for NMs and NMs mixtures. In particular, careful thought and validation is needed for NMs-specific molecular initiating events (MIEs), given that NMs can induce both physical and chemical effects, and that in the case of NMs mixtures an MIE might be associated with an adsorbed co-pollutant whose bioavailability is increased through uptake with the NM.
- There is an urgent to further develop ontologies, public databases and user-friendly nanoinformatics tools for modelling and predicting these complex interactions between NMs and environmental contaminants during co-exposure assessments. This is an active area of development, with recent developments in this direction including the NanoPharos database [268] designed for computational modelling of NMs. Collectively, these tools and approaches support the implementation of the FAIR data principles in the nanoecotoxicology research community to facilitate data-driven science, predictive mixture ecotoxicity and risk assessment.

## CRediT authorship contribution statement

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. D.S.T.M., E-J.A.E. G.H.S., R.P., A.M.Z.M., H.H.D., A.G.P., and G.M.: literature research, writing, and editing. D.S.T.M., A.F., A.A. and I.L.: funding acquisition, supervision, project administration, and writing. All authors contributed to the article and approved the submitted version.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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