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## Emerging investigator series: metal nanoparticles in freshwater: transformation, bioavailability and effects on invertebrates†

Ting Wang and Wei Liu \*

The increasing use of metal oxide-based nanoparticles (MNPs) and their release into the environment cast concerns about their environmental impacts. Massive efforts have been focused on environmental behaviours and ecotoxicities to figure out the potential threats posed by MNPs. This review systematically summarises and re-analyses published data about the MNP interactions and transformation processes in freshwater and the toxicological effects of MNPs on invertebrates. A case study was conducted through meta-analysis to examine the impacts of silver nanoparticle exposure to freshwater invertebrates. The conclusions categorized the current understanding of the outcome and ecotoxicity of MNPs in freshwater. The adverse outcome pathway (AOP) is recommended for environmental risk assessment as it provides a rapid and accurate risk assessment of an increasing number of novel compounds consuming fewer resources and animal tests. Invertebrates contribute significantly towards developing robust AOPs thanks to a shorter life cycle, allowing chronic and complete life cycle toxicity tests.

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### Environmental significance

MNPs may undergo different environmental processes in the aquatic system, consequently changing their mobility, bioavailability, and toxicity to organisms. This review summarises and re-analyses published data regarding the MNP interaction with environmental factors and transformation processes in freshwater and the toxicological effects of MNPs in three major groups of invertebrates by considering the bioavailability of MNPs as an essential step to understand their biological outcome. After ingestion by freshwater invertebrates, MNPs are likely to be accumulated in sensitive organs and induce ROS production, a predominant mechanism leading to toxicity. ROS production induced by MNPs is controlled by size, shape, surface, composition, solubility, aggregation and particle uptake. In addition, a meta-analysis was conducted to examine the impacts of silver nanoparticle exposure on freshwater invertebrates as a case study. Significant research gaps and recommendations for future research are also indicated.

## 1. Introduction

Nanoparticles (NPs), with at least two dimensions between 1 and 100 nm, possess physicochemical properties that offer many medical, societal and technological benefits.<sup>1</sup> Metal and metal oxide-based NPs (MNPs) are the most commonly used materials and are particularly important to our life.<sup>2</sup> For example, the Organization for Economic Co-operation and Development (OECD) has highlighted silver (Ag), zinc oxide (ZnO), titanium dioxide (TiO<sub>2</sub>) and cerium dioxide (CeO<sub>2</sub>) NPs as high interest due to their widespread applications and inherent properties.<sup>3</sup> However, the mass application of

products containing NPs inevitably results in nanoparticle pollution, which triggers concerns about their environmental impacts.<sup>4,5</sup> Water ecosystems are among the most vulnerable to contamination because they receive and accumulate large amounts of pollutants, including nanomaterials, from rainfall, surface runoff, subsurface seepage or wastewater discharge.<sup>6</sup> As the primary water environment in inland areas, freshwater ecosystems are undoubtedly the prime victim of nanomaterial pollution. The occurrence of NPs in the freshwater environment is globally observed.<sup>7,8</sup> Numerous research studies show that the effects of nanomaterials on freshwater organisms exist at all biological levels and all stages of the organism's life cycle.<sup>9–11</sup>

Aquatic invertebrates represent well-established model organisms for MNP toxicological studies.<sup>12</sup> Some invertebrates, including bivalves,<sup>13,14</sup> gastropods<sup>15,16</sup> and crustaceans,<sup>17,18</sup> are considered good environmental quality indicators due to their wide geographic distribution,

Department F.-A. Forel for Environmental and Aquatic Sciences, Faculty of Sciences, Earth and Environment Sciences, University of Geneva, Uni Carl Vogt, 66 Blvd Carl-Vogt, CH 1211 Geneva, Switzerland. E-mail: wei.liu@unige.ch  
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abundant numbers and easy availability. Bivalves (*e.g.*, *Mytilus* spp.) are filter-feeding invertebrates in natural waters with highly developed endo- and phagocytosis pathways.<sup>14</sup> Based on pollutant accumulation in these organisms, fitness can be adopted as a biological indicator of the load on the ecosystem.<sup>13</sup> Gastropods (*e.g.*, *Lymnaea stagnalis*) display significant advantages in high sensitivity and susceptibility to water contamination and straightforward laboratory maintenance.<sup>16</sup> Such organisms are also an ethically acceptable alternative to an animal model in toxicity tests.<sup>15,19</sup> Regarding crustaceans, *Daphnia magna* is one of the most sensitive organisms widely used and included in several guidelines and international standards for acute and chronic tests.<sup>17,18</sup> It can thus be concluded that aquatic invertebrates are very promising for ecotoxicology research.

How MNPs affect freshwater ecosystems has drawn increasing attention in the last few years. One of the major concerns is the bioavailability of MNPs in freshwater organisms, which may undermine their fitness. Compared with marine water, there is more natural organic matter (NOM) in freshwater, which rapidly adsorbs onto the surface of MNPs, producing repulsive forces between particles and decreasing their aggregation.<sup>20</sup> Furthermore, freshwater normally displays lower ionic strength (conductivity: 0.03–0.10 mS cm<sup>-1</sup>) than marine water (47.8–49.8 mS cm<sup>-1</sup>), where charged NPs are less neutralised by counterions and keep their colloidal stability and thus are less aggregated.<sup>21</sup> This might lead to a longer residence time (which is related to the bioavailability) of NPs in freshwater rather than in marine waters.<sup>20</sup> Many existing documentaries on the bioaccumulation of various MNPs, including AuNPs,<sup>22,23</sup> AgNPs,<sup>24,25</sup> and CuONPs are available.<sup>26,27</sup> MNPs are primarily mediated by ingestion and subsequently accumulate in gut tissues of organisms and are often not readily taken up by the epithelial surface and transported *via* the circulatory system.<sup>28,29</sup> Further, MNPs may be taken up by plasma membrane vesicles rather than being absorbed by the cell through transferring proteins and passive diffusion. Once ingested, MNPs remain in the digestive tracts of organisms for days up to weeks, allowing the transfer of NPs to the food web.<sup>30–32</sup> Biomagnification may cause more significant impacts on freshwater ecosystems because it amplifies the concentrations of MNPs at upper trophic levels by an order of magnitude.<sup>30</sup> Thus, understanding the capacity for MNPs to bioaccumulate in organisms and subsequently transfer through and biomagnify within food chains is crucial.

Another primary concern is how MNPs impact freshwater organisms after ingestion. Multiple recent reviews have summarized the MNP's toxicity to aquatic organisms and highlighted that additional research to improve our understanding of the adverse impacts of MNPs is necessary. The physicochemical characteristics of MNPs change as an environmental release with time under the influence of the surrounding environment, thereby affecting the impact on organisms. Abiotic factors such as media composition,

sulfidation, irradiation, pH and ionic strength (IS) of media may also contribute to determining the outcome and toxicity of NPs.<sup>33</sup> The overall potential adverse impacts of MNP pollution are considered challenging to predict. We need to integrate quantitative studies to produce a more comprehensive and objective evaluation of the differential biological responses triggered by MNP exposure.

A meta-analysis is a powerful tool to rigorously assess the findings of published independent research, which can help determine the effect size for result variables. A meta-analysis has been developed to study the toxicological TiO<sub>2</sub> NPs on marine bivalves.<sup>34,35</sup> Furthermore, due to the diversity of the MNPs, toxicity evaluation is challenging to access, and the limitation remains to test various MNPs' toxicity each time. To address these limitations, a toxicity mechanism-based approach, such as the adverse outcome pathway (AOP), is more practical than a substance-based approach. However, the majority of NP toxicity studies focused on ecotoxicity took apical endpoints, and only a few dealt with toxicity mechanisms.<sup>36–39</sup> AOPs span numerous levels of biological organization, from the molecular level to an organism level or even to population levels for ecotoxicology scenarios.<sup>40</sup> Since the AOP is an evidence-based framework, it is constantly updated following new evidence of toxicity mechanisms, improving its reliability and application.<sup>41</sup> It also allows us to identify data gaps for future research based on the current AOP. Thus, although the study of toxicity mechanisms of MNPs is limited, identifying the currently available molecular-level toxicity information and linking it with AO is a tactical beginning for the risk assessment of MNPs.

This review summarizes the transformation processes that MNPs undergo and the effects of main physical–chemical properties and environmental factors on MNPs' transformation in freshwater. We present the records that examined the impact of MNPs on freshwater invertebrates, experimentally and under laboratory-controlled conditions. Based on these studies, we conduct a meta-analysis to investigate the importance of the MNP type, size, coating, concentration and exposure time effect of MNPs on freshwater invertebrates. Through the comprehensive analysis of the current research on the effects of MNPs, we propose an appropriate AOP to manage MNPs based on the existing literature and highlight research gaps, discuss methods, and identify recommendations and perspectives for future research.

## 2. Environmental transformations of MNPs in freshwater

Knowledge of the environmental fate and behaviour has essential implications in evaluating the MNPs' hazards and ecological and environmental risks. When NPs are released into the environment, these NPs, which have high surface activity, readily interact with substances in complex environments, causing changes in their surface structure,



resulting in more unique environmental behaviours.<sup>42</sup> The MNPs exposed to freshwater systems undergo various physical (e.g., aggregation, adsorption and sedimentation), chemical (e.g., dissolution and sulfidation), and biological (e.g., eco-bio-corona) processes.<sup>9,33,43</sup> The above transformations are known to depend on the physicochemical properties (e.g., size, charge, surface coating, concentration) of MNPs and water conditions (e.g., IS, pH, NOM).<sup>43,44</sup>

## 2.1 Physical transformations

**2.1.1 Aggregation.** Aggregation refers to MNP cluster formation in suspension, and such a process increases the NPs' size and density, which leads to gravitational settling in the sediment and impact on benthic organisms.<sup>45,46</sup> NPs' size has been found to affect the aggregation, due to the higher percentage of atoms present on the surface of NPs with smaller size, resulting in surface chemistry change and subsequent layer charge decrease, thus promoting this process.<sup>47</sup> For instance, in moderately hard water, smaller platinum (Pt) NPs (20 and 30 nm) formed a wide-size range and larger aggregates than larger sizes (75 nm).<sup>48</sup> Similar results were also found in AuNPs when exposed to a sterile lake water medium, where 30 nm NPs aggregated more rapidly than 40 nm NPs.<sup>49</sup> Generally, higher concentrations carry higher surface charges, which promotes the stability of NPs by limiting the inter-particle contact. For example, Al<sub>2</sub>O<sub>3</sub>NPs showed lower aggregation at 1 mg L<sup>-1</sup> concentration compared with lower 0.1 mg L<sup>-1</sup> in river water.<sup>50</sup>

The nature of the surface coating also acts as an essential factor in deciding the MNPs' aggregation. In lake water, compared with polyvinylpyrrolidone (PVP)-coated AgNPs, lipoic acid (Lip) and citrate (Cit) coatings demonstrate lower protective effects of AgNPs against aggregation.<sup>51</sup> These results illustrate that the surface coating stabilising AgNPs (i.e., PVP, higher affinity) by steric repulsion is more effective than electrostatic repulsion (i.e., Cit and Lip, weak affinity). In both raw and filtered river water, polyethylene glycol (PEG) or carboxylated PEG (PEG-COOH)-coated AuNPs showed good stability, while branched polyethyleneimine (bPEI), amine-functionalized PEG (PEG-amine) and Cit-coated AuNPs have been shown to readily aggregate.<sup>52</sup> These neutral or negatively-charged coatings (e.g., PEG, PEG-COOH-) could prevent homoaggregation of NPs *via* electrical double-layer (EDL) compression and are not affected by the presence of NOM in test medium.<sup>53</sup> Although positively charged (e.g., bPEI, PEG-amine-, Cit-) coatings could also serve to stabilize the NPs, they are more easily adsorbed with NOM and promote aggregation either by interparticle bridging or divalent cation bridging.<sup>52,53</sup>

The point of zero charge (pH<sub>PZC</sub>) refers to the pH when the net surface charge of NPs approaches zero. Theoretically, the smaller the difference between solution pH and pH<sub>PZC</sub>, the greater the aggregation rate. For example, in wastewater,

when the pH value approaches the pH<sub>PZC</sub> of ZnONPs, due to the decreased repulsive interactions between NPs, the process of aggregation and deposition occurs within two hours.<sup>54</sup> When the pH value of simulated lake water reached the pH<sub>PZC</sub> of TiO<sub>2</sub>NPs, large agglomerates were observed.<sup>55</sup> In general, divalent electrolytes (Ca<sup>2+</sup> and Mg<sup>2+</sup>) in freshwater could destabilize MNPs, and the enhanced IS tends to weaken electrostatic repulsion between particles, compressing the EDL surface and leading to aggregation.<sup>56,57</sup> For instance, CuNPs' aggregate size correlated well with IS in ground water and freshwater.<sup>56</sup> An increase in the particle size of AgNPs was reported in surface water with higher IS.<sup>57</sup> After exposure to filtered river water, the AgNPs aggregate readily within one hour.<sup>58</sup> Under freshwater relevant conditions, the aggregation level of Cit-AgNPs and TiO<sub>2</sub>NPs depends mainly upon the concentration of Ca<sup>2+</sup>.<sup>59</sup>

In freshwater, NOM represents the most critical ligand group, composed mainly of humic acids (HAs) and fulvic acids (FAs). Rich functional groups of NOM provide the high potential to adsorb MNPs *via* various mechanisms, including hydrophobic interactions, van der Waals interactions, surface ion chelation, cation bridging, *etc.*<sup>42,60</sup> It is suggested that HAs can inhibit the aggregation and deposition of MNPs (e.g., Ag, Fe, Fe<sub>3</sub>O<sub>4</sub>, Al<sub>2</sub>O<sub>3</sub>, TiO<sub>2</sub>, SiO<sub>2</sub> and ZnO) through electrostatic and spatial dislocation effects.<sup>42</sup> However, in the presence of divalent cations, dissolved organic matter (DOM) can flocculate on the surface of MNPs through cationic bridging, causing the occurrence of aggregation and deposition of MNPs.<sup>61</sup> In addition, different pH and ionic conditions affect the adsorption of NOM. For example, under acidic and basic conditions, DOM can be adsorbed on the surface of AgNPs *via* carboxyl groups and aliphatic and phenolic groups, respectively.<sup>62</sup> In the presence of NOM, the larger-sized PVP-PtNPs (95 nm) are more affected than the smaller NPs (20 nm), which form larger agglomerates in artificial freshwater.<sup>48</sup> Similar results were observed in FA coated CeO<sub>2</sub>NPs, which generated small aggregates in lake water, while large aggregates were obtained in the absence of FAs.<sup>63</sup> In natural lake water, NOM could accelerate the heteroaggregation of TiO<sub>2</sub>NPs and subsequent sedimentation.<sup>64</sup>

**2.1.2 Sedimentation.** The sedimentation process is critical for removing MNPs from water bodies and thus is essential in their fate studies.<sup>65</sup> For example, after exposure to moderately hard water for 24 hours, around 28–53% of PtNPs were likely to settle out of the suspension.<sup>48</sup> Homoaggregation (MNPs' cluster) and heteroaggregation (MNPs associated with suspended natural colloids) of MNPs lead to denser particles and are considered the main pathways for deposition in the sediment.<sup>33</sup> The prevalence of NOM and IS in an aqueous environment are considered two key factors affecting NPs' sedimentation.

NOM can promote aggregation and aggravate sedimentation by bridging the function at low concentrations while inhibiting or slackening this process by increasing the surface charge and spatial resistance at high concentrations.



For instance, in freshwater, almost complete sedimentations were observed for CuNPs, AlNPs and MnNPs within less than 15 min due to rapid aggregation.<sup>66</sup> Furthermore, a higher concentration of NOM (*e.g.*, HA and dihydroxy benzoic acid) enhanced the NPs' electrostatic stabilization, evidenced by lower sedimentation velocity.<sup>66</sup> IS could promote sedimentation by compressing the EDL of NPs.<sup>56</sup> For example, sedimentation of CuNPs and CuONPs were directly or inversely proportional to IS and organic content in lake water, respectively.<sup>56</sup> A recent study highlights that heteroaggregation between AgNPs and suspended sediment (SS) played a predominant role in settlement behaviour under high IS conditions, while AgNPs distributed in the overlying waters under low-salinity and SS conditions.<sup>45</sup> Similarly, CeO<sub>2</sub>NPs were stable in low pH, IS, and SS water, whereas aggregation occurred with increasing cation concentration, showing that the larger size NPs (>1000 nm) settle quickly to the bottom while the smaller particles are suspended in solution.<sup>67</sup>

## 2.2 Chemical transformations

**2.2.1 Dissolution.** MNPs readily react with H<sup>+</sup> and dissolved oxygen from water and release metal ions, a process usually referred to as dissolution.<sup>33</sup> Some MNPs with active chemistry properties, such as AgNPs, CuONPs and ZnONPs, are susceptible to oxidation dissolution.<sup>33</sup> In the case of AgNPs, Ag<sup>+</sup> could be released *via* the redistribution of adsorbed Ag<sup>+</sup> on the NPs' surface during the synthesis process and dissolution of the outer Ag<sub>2</sub>O oxide layer.<sup>68</sup>

Smaller NPs dissolve more quickly than larger ones due to the enhanced surface area, indicating that more available surface sites are involved in dissolution.<sup>69</sup> For instance, in lake water medium and freshwater-like conditions, enhanced dissolution was found at smaller-sized (50 nm) ZnONPs than 100 nm and bulk form.<sup>70,71</sup> In media relevant for freshwater, the smallest AgNPs (5 nm) promoted a higher dissolution rate than larger NPs (10 and 20 nm), which could be explained by the proton number that active molecular oxygen adsorbed by the surface.<sup>72</sup> In freshwater, a significant dissolution percentage (~81.98%) was found at lower concentrations (10 mg L<sup>-1</sup>) of ZnONPs compared with 1000 mg L<sup>-1</sup>, which exhibited ~78.83% dissolution after one hour of exposure.<sup>73</sup> Similar results were found in AgNPs after exposure to moderately hard reconstituted water, where dissolution rates of 5 µg mL<sup>-1</sup> are much higher than that for 100 µg mL<sup>-1</sup>.<sup>74</sup> This difference might be explained by the higher ratio of Ag<sup>+</sup> to Ag complexing agents, which helps scavenge Ag<sup>+</sup>, or Ag<sup>+</sup> could associate back with the NPs at high concentrations.<sup>74</sup>

The surface coating of NPs and test media also affects its solubilisation pattern. For some highly soluble MNPs (*e.g.*, Ag, CuO, and ZnONPs), their dissolution rates showed a wide range of 1–80% under various environmental scenarios, which demonstrates the critical role of media constituents (*e.g.*, pH, IS and NOM) in this process.<sup>75</sup> For example, Cit-AgNPs showed

higher dissolution when compared to PVP AgNPs<sup>76,77</sup> in laboratory scenarios, where PVP-AgNPs are more prone to Ag<sup>+</sup> release than Cit-AgNPs in natural waters.<sup>78</sup> Selenium (Se) NPs displayed a higher dissolution rate (~35.3%) in lake water when compared with ultrapure water (~20.8%).<sup>79</sup> The presence of NOM in freshwater reduces the dissolution of CuONPs, which might be *via* metal ion-chelating or coating particle surfaces.<sup>56</sup> In synthetic freshwater, the HAs and dihydroxy benzoic acid (DHBA) could adsorb on CuNPs, AlNPs and MnNPs within one minute and, in particular, enhance the dissolution of AlNPs and CuNPs.<sup>66</sup> The HA and DHBA could coordinate with the NP surface *via* forming mononuclear surface complexes, which weaken the bonds between the metal and oxygen in the surface oxide and thus promote the dissolution of CuNPs.<sup>80</sup>

**2.2.2 Sulfidation.** Sulfidation plays an essential role in controlling metal ion concentration in the environment due to the high complexation tendency with sulfide ligands.<sup>33</sup> Sulfide, which is generated by microbial sulfate reduction, commonly exists in hypoxic environments such as riverine, lake sediments and wastewater treatment plants.<sup>81,82</sup> For instance, sparingly soluble silver sulfides (Ag<sub>2</sub>S) have been identified as a major Ag species in the sewage sludge taken from urban wastewater systems.<sup>81</sup> In moderately hard reconstituted water, AgNPs could transform to Ag<sub>2</sub>S *via* direct or indirect oxysulfidation, depending on the concentration of sulfides.<sup>83</sup> At high concentration (mg L<sup>-1</sup>) applied, the AgNPs undergo a fast, direct NPs–fluid reaction and generate Ag<sub>2</sub>S; meanwhile at low concentration, the AgNPs first develop into silver ions before reacting with sulfide ions and eventually generate Ag<sub>2</sub>S.<sup>83,84</sup> Such a transformation process could be reversible, and one recent study proposed that Ag could be remobilized from Ag<sub>2</sub>S with the aid of Fe(III) in freshwater under light conditions.<sup>85</sup>

Generally, smaller sizes could enhance the sulfidation rate of AgNPs, probably due to the dependency of the reaction rate on the specific surface area of NPs.<sup>86</sup> The enhanced ratio of HS<sup>-</sup>/Ag also contributes to the sulfidation of AgNPs, where Ag<sub>2</sub>S bridges are formed between NPs.<sup>86</sup> Other factors, such as NOM, can also affect the sulfidation process. For example, the presence of HAs and FAs could slightly enhance or decrease the sulfidation rate of AgNPs, respectively.<sup>38</sup> HAs might promote the sulfidation reaction *via* replacing the surface coating of NPs, giving rise to an extensive available surface area, while FAs diminish this process by blocking the surface of AgNPs. Notably, the sulfidation process is usually accompanied by more significant aggregation and sedimentation and a lower dissolution profile, which might influence the fate and bioavailability of NPs (*e.g.*, AgNPs, ZnONPs).<sup>9,86</sup> For instance, a recent study highlighted that the sulfidation process could diminish the toxicity of AgNPs in constructed wetlands.<sup>87</sup>

## 2.3 Biological transformations

The eco-bio-corona is the principal biological mediated transformation in the environment. Upon entering the



aquatic environment, MNPs are rapidly encapsulated by biomolecules (e.g., extracellular polymeric substances (EPS), which are secreted mainly by aquatic organisms' metabolic activities), forming an eco-corona. Similarly, the bio-corona is formed *via* interacting with the adsorbed endogenous proteins in the presence of organisms.<sup>88,89</sup> The eco-bio-corona can modify the distribution, accumulation, degradation, intracellular recognition and biotoxicity of NPs by altering their structure, kinetic behaviour and function.<sup>90</sup> On the other hand, the biocompatibility of NPs within organisms can be improved by modulating the cellular uptake of NPs. For example, the bio-corona can control the interaction of NPs with outer membrane receptors for specific cellular uptake.<sup>91</sup>

Biomolecules have been known to bind with metals *via* electrostatic interaction and complexation, with the aid of many functional groups, including carboxyl, hydroxyl, *etc.*<sup>92</sup> EPS are amphiphilic molecules with a hydrophobic region, facilitating adsorption onto organic substances. Studies have shown that positively charged NPs generally readily interact with negatively charged EPS such as polysaccharides or proteins, while hydrophobic components of EPS can act as stabilisers to stabilise NPs. For example, the EPS of freshwater biofilms could stabilize CeO<sub>2</sub>NPs and induce aggregation of AgNPs.<sup>93</sup> Similarly, EPS adsorption enhanced the ZnONPs' stability with electrostatic attraction and surface complexation involved.<sup>94</sup> EPS also exhibit reducing characteristics due to the reducing functional groups (*i.e.*, hydroxyls, phenolic-OH, thiols and aldehydes).<sup>95</sup> Recent research proposed that Ag<sup>+</sup> could be reduced to AgNPs by EPS in natural water, and this process could be enhanced under light irradiation.<sup>96</sup> Thus, EPS can change the environmental behaviour of NPs and might subsequently influence their fate and toxicity in the environment.<sup>39</sup>

In addition to the EPS derived from natural water, the biomolecules secreted by organisms could also interact with the MNPs' surface and form a new identity (*i.e.*, eco-corona) which affects the stability and toxicity of the MNPs toward organisms in the surrounding environment. For example, a recent study showed that AuNPs could interact with protein secreted by *D. magna* and produce protein-corona, reducing the AuNP aggregation and potentially detoxifying AuNPs to *D. magna* by shielding their surface attraction.<sup>97</sup> Conversely, for polystyrene NPs, a previous study highlighted the promoted aggregation of NPs with protein-corona in a dispersion medium previously conditioned with *D. magna* neonates.<sup>98</sup> Many studies reviewed the bio-corona formation and associated biological effects.<sup>91,99,100</sup> However, most of these focused on biomedical and human toxicology, and studies demonstrating this field in natural waters, including freshwater, are still elusive.<sup>99,100</sup>

To sum up, after entering the freshwater system, MNPs undergo physical, chemical, and biological transformational processes governed by NPs' characteristics and the chemical properties of water. In general, small size NPs easily form large clusters due to the large surface and high surface energy.

Therefore, the concentration and surface coating of NPs demonstrate essential roles in determining the size by electrostatic and steric repulsion functions. The pH impacts the surface potential of NPs, and large clusters are formed as the pH approaches the p*H*<sub>PZC</sub>. The low IS and high NOM concentration in freshwater generally stabilize the NPs but the fate and behaviour of NPs are also affected by other factors due to the complex composition of the natural system. A schematic diagram showing the environmental transformations MNPs in aquatic environments is presented in Fig. 1.

### 3. Bioavailability of MNPs in freshwater invertebrates

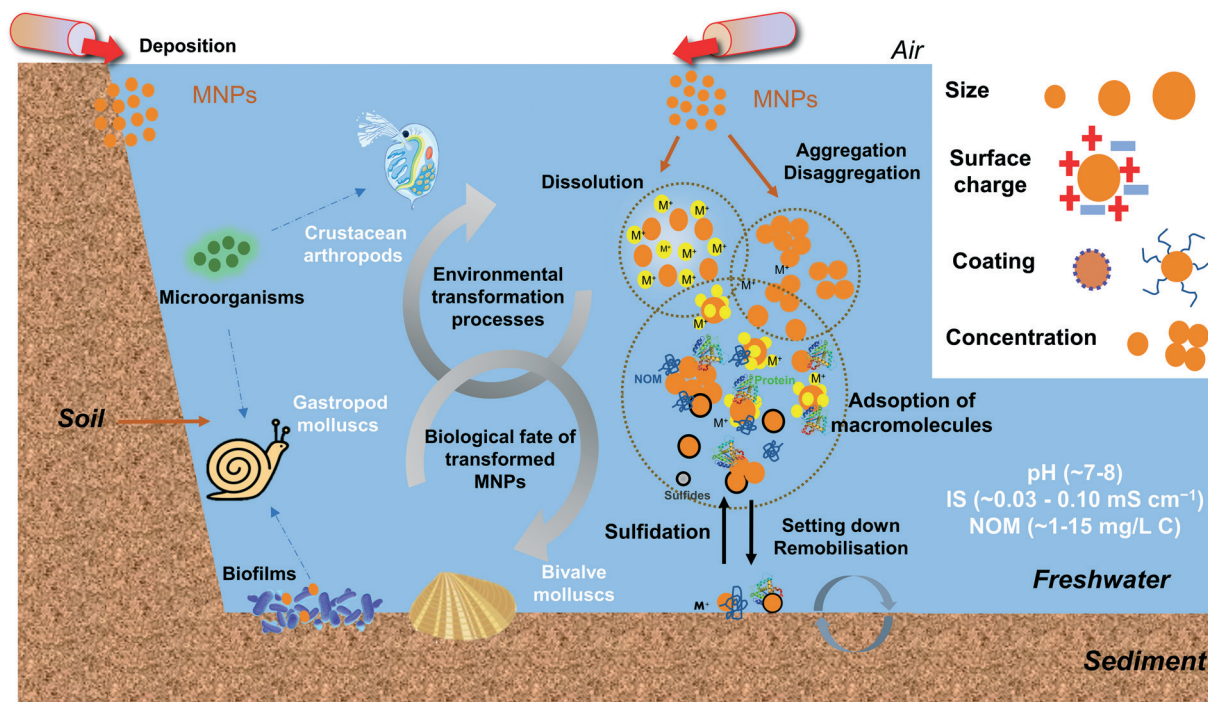
Studies on bioavailability and uptake are critically essential to link the environmental transformation of MNPs to biological responses.<sup>101</sup> Individual free MNPs, homoaggregates and MNP heteroaggregates can accumulate and subsequently remain within organisms.<sup>18,32,102–104</sup> In natural water, some soluble MNPs (e.g., ZnONPs, CuONPs and AgNPs) can release metal ions, which can cause latent free-ion toxicity, resulting in different toxic impacts than exposure to the pristine MNPs.<sup>18,105,106</sup> Understanding the bioaccumulation of MNPs is pivotal for addressing their ecotoxicity and risk assessment because it determines the potential MNP concentration. Since mollusk and Arthropoda are the largest phyla of invertebrate animals, three commonly used classes for toxicology studies (bivalvia, gastropoda and crustacean arthropods) are discussed in separate sections.

#### 3.1 Bivalve molluscs

Bivalve molluscs, sedentary-style filter-feeding invertebrates, are widely adopted as bioindicators in nanotoxicology.<sup>107</sup> Filter-feeding bivalves may filter large water throughputs at high rates for respiratory and nutritional purposes and thus may ingest considerable amounts of MNPs dispersed in the water.<sup>3</sup> Bivalves are considered an effective sink for MNPs.<sup>108</sup> Suspended MNPs from the water, concentrated in faeces and pseudo-faeces, can be captured and ingested by benthic invertebrates and transferred into the aquatic food chain.<sup>109</sup> As filter feeders, bivalves represent one of the most important pathways of MNPs into the human food chain.<sup>35,110</sup>

Bioaccumulation tests of MNPs in freshwater bivalves have primarily been conducted in *Corbicula fluminea*,<sup>22,102</sup> *Anodonta cygnea*,<sup>111</sup> and *Dreissena polymorpha*.<sup>112,113</sup> After exposure to AgNPs, most particles accumulated in the viscera (gills and digestive tract) of *C. fluminea*, while mantle, muscle, and foot tissue showed low accumulation potential.<sup>102</sup> This indicates no significant transport of AgNPs through the different compartment tissues or hemolymph and thus points to the negligible bioavailability of AgNPs to *C. fluminea*. Gills and digestive glands are considered preferential organs of MNP accumulation in filter-feeding organisms, compared to mantle and foot organs.<sup>114</sup> *A. cygnea* in exposure to sub-lethal concentration of CuONPs (40 nm) for 12 d leads to a significant





**Fig. 1** The schematic diagram illustrates the overview of the environmental transformations and the biological fate of transformed MNPs in freshwater environments. MNPs may undergo physical, chemical, and biological transformation processes. The MNP transformation differs mainly according to their intrinsic properties (e.g., size, surface charge, coating and concentration) and environmental conditions (e.g., pH, IS, NOM, related values are referenced from ref. 21, 56 and 64). The transformed MNPs may accumulate in some typical freshwater invertebrates, such as filter-feeding bivalve molluscs, gastropods molluscs and crustacean arthropods, and contribute to the trophic transfer of MNPs.

accumulation of Cu in mantle and foot than that in gill.<sup>111,115</sup> It is worth noting that the MNP's surface properties could influence the bioaccumulation process. *D. polymorpha* accumulated Cit-CeO<sub>2</sub>NPs three times more than bare-CeO<sub>2</sub>NPs; the reason behind this difference is correlated with their distinct behaviours presented in the water column. Cit-CeO<sub>2</sub>NPs are more stable than bare-CeO<sub>2</sub>NPs in water, making it easier to be captured by mussels.<sup>112</sup>

Freshwater bivalves can directly ingest and rapidly accumulate MNPs in water (i.e., bioconcentration)<sup>102,111,112</sup> or intake of MNPs *via* trophic transfer (e.g., from prey to predator).<sup>22</sup> For example, in Arini's study,<sup>22</sup> the rate constants for uptake of AuNPs from water (*kuw*) and food (*kuf*) were accessed. The former way is likely to be the primary exposure pathway for *C. fluminea*, considering the higher *kuw* than *kuf*. During the dietary exposure, *C. fluminea* decreased its ventilator activity, resulting in reduced gill filtration. Thus, Au accumulation presents a 30-fold reduction after dietary exposure to algae loaded with AuNPs compared to waterborne exposure.<sup>22</sup> The results above revealed that MNPs might compromise bivalves' feeding capacity, but indeed, not just for bivalves, other freshwater species were also observed to exhibit filtration behaviour impairments as reviewed below.

### 3.2 Gastropod molluscs

Gastropod organisms are ubiquitous in the aquatic ecosystem. Most freshwater gastropods (e.g., *Lymnaea*

*stagnalis*) are lung-breathing pulmonates; hence, they spend time at the water surface for air-breathing and ingestion.<sup>116</sup> They are considered sentinel species for pollution biomonitoring in their wide geographic distribution, relatively sedentary life habits, and ease of availability.<sup>117</sup>

Current studies have indicated that the dissolved metal ion form (e.g., Ag<sup>+</sup>, Cu<sup>2+</sup>) was more bioavailable than their nanoparticulate forms in gastropod molluscs.<sup>24,27</sup> For example, the total body burden of Cu in the whole freshwater snails *Potamopyrgus antipodarum*<sup>118</sup> and *Bellamya aeruginosa*<sup>27</sup> was significantly higher for those exposed to Cu<sup>2+</sup> than both control and CuONPs treatments. Freshwater snails *B. aeruginosa*<sup>24</sup> and *Peringia ulvae*<sup>119</sup> also accumulated higher levels of Ag from Ag<sup>+</sup> than from particulate Ag. This ingestion discrepancy could be related to the different uptake pathways between MNPs and dissolved metal ions. The former could be potentially internalized *via* endocytotic pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, or macropinocytosis. Meanwhile the dissolved ions from MNPs can enter cells *via* transporter channels, including the proton-coupled Na<sup>+</sup> channel.<sup>120</sup> Furthermore, MNPs tend to agglomerate, aggregate and form a bio-ecorona in natural water, and thus their bioavailability would be substantially reduced.<sup>89,121</sup> However, some research showed the contrary. For example, *B. aeruginosa* accumulated a higher concentration of Ag from sediments spiked with Ag<sup>+</sup> than AgNPs.<sup>24</sup> Upon the same total metal sediment concentrations, CuO NPs were more bioavailable than



aqueous Cu to deposit-feeding snail *P. antipodarum*,<sup>122</sup> which is inconsistent with bioaccumulation results with *B. aeruginosa*.<sup>27</sup> This discrepancy may result from differences in experimental design, particularly the exposure duration (56 d for the former vs. 28 d for the latter) to CuONPs. The elimination rate of CuONPs may be lower than that of dissolved Cu<sup>2+</sup> over time. In addition, no significant Cu accumulation difference in *Planorbarius corneus* was found between matrix-embedded CuO NPs and controls with realistic low concentrations, which might make detecting Cu from the matrix and biological tissues more complicated.<sup>123</sup>

The physicochemical properties of the exposure media (e.g., NOM) can also affect the MNP bioaccumulation. Sikder *et al.* indicated the importance of (PtNPs) size and interfacial interactions with NOM on Pt bioavailability.<sup>48</sup> Both dissolved and PtNPs (size range from 20 ~95 nm) show good bioavailability to *L. stagnalis*. In the absence of NOM, the larger Pt NPs have higher bioavailability than the smaller ones, while it is the opposite in the presence of NOM.<sup>48</sup> This may be explained by the surface adsorption of NOM on larger particles, making them susceptible to rapid precipitation and forming larger agglomerates, and suggests that PtNPs' *in vivo* transformation could have higher adverse effects on organisms than the dissolved metal ion form.

Diet-borne exposure plays a significant role in the bioaccumulation and toxicity of NPs in aquatic organisms, compared with single waterborne scenarios.<sup>105,111,124–127</sup> For example, dietary exposures to ZnONPs and AgNPs suppressed the assimilation efficiency of the snail *L. stagnalis*.<sup>125,128</sup> The reduced bioavailability of MNPs may be explained by the agglomeration/aggregation of the MNPs onto diatom mats or detrimental effects on digestion.<sup>125</sup> Recent work highlights the higher Ag bioaccumulation potential in the Ag<sup>+</sup> treatment compared with Ag<sub>2</sub>SNP exposures, but no biomagnification was observed from the freshwater snail *Physa acuta* to the planarian *Girardia tigrina*.<sup>129</sup> To fully mimic the real environmental scenario with a food chain with more than three species, another study constructed a freshwater ecosystem (including clam *C. fluminea*, snail *P. acuta*, and water flea *D. magna*) and determined the bioaccumulation and biomagnification of CeO<sub>2</sub>NPs *via* long-term exposure. Results found that bioaccumulated Ce in all tested species was negatively correlated with its trophic level, showing no biomagnification of CeO<sub>2</sub>NPs through this food web.<sup>32</sup> Conversely, the significant biomagnification of CeO<sub>2</sub>NPs was reported in terrestrial food chains.<sup>130</sup> These findings implied that biomagnification of NPs in the natural environment might be complicated and affected by several factors, such as NPs' intrinsic characteristics and prey-predator dependence.<sup>131</sup> Future studies addressing mechanisms underlying the trophic transfer of NPs are urgently needed.

### 3.3 Crustacean arthropods

Current studies about MNP bioavailability in freshwater crustacean taxa have mainly focused on *Daphnia* spp.

*Daphnia*, the common water flea, is widely used as a model aquatic organism in toxicity studies.<sup>132</sup> So far, MNP accumulation studies have been mainly performed with TiO<sub>2</sub>-NPs,<sup>133–136</sup> AgNPs<sup>25,137,138</sup> and ZnNPs.<sup>103</sup>

Current studies have indicated that MNPs (e.g., TiO<sub>2</sub>NPs and CuONPs) mainly accumulated in the gut tissue of *D. magna*.<sup>18,133</sup> As filter feeders, *D. magna* can trap MNPs inside the body upon filtration and appendage movement of *D. magna* can also contribute to NP ingestion if there was NP adherence to the carapace.<sup>18</sup> Fan *et al.* investigated the effects of TiO<sub>2</sub>NPs' properties on the BCFs for the first time.<sup>136</sup> They reported a higher BCF at lower concentrations (0.1 and 1 mg L<sup>-1</sup>) than at a higher dose (10 mg L<sup>-1</sup>) regarding TiO<sub>2</sub>NP exposure in *D. magna*.<sup>136</sup> Under a higher concentration scenario, TiO<sub>2</sub>NPs might form larger aggregates and thus not be taken up by *D. magna*.

The diet has been demonstrated as the most critical route of uptake and bioaccumulation for trace metals. Exposure to MNP contaminated diet can suppress the feeding behaviour in *Daphnia* spp. Previous findings suggest that *Daphnia* spp. decrease AgNP intake during dietary exposure.<sup>137</sup> Possible explanations for the feeding rate reductions may include the accumulation of MNPs in the gut or higher sedimentation of contaminated algae to the bottom of the container resulting in lower availability for filter-feeding.<sup>139</sup> AgNPs were not eliminated from *Daphnia* over the depuration period (48 h), leading to further possible transport of AgNPs along the food chain.<sup>25</sup> Indeed, it is well established that MNPs, such as AgNPs,<sup>137</sup> TiO<sub>2</sub>NPs<sup>133–135</sup> and AuNPs,<sup>140</sup> can be transferred from freshwater algal diet to *Daphnia* spp. Moreover, the trophic transfer showed size dependence in the Chen *et al.* experiment<sup>133</sup> which found that TiO<sub>2</sub>NPs' biomagnification factors (BMFs) decreased with increasing MNP size. Generally, due to the higher specific surface area, smaller TiO<sub>2</sub>NPs are more prone to attach to the algal cell than larger ones, causing the latter to be of lower bioavailability.<sup>133</sup>

To summarize, the bioavailability of MNPs relates to the subsequent toxicity to aquatic organisms; thus it is crucial to know the accumulation of MNPs in organisms to understand and predict their toxicity. The high potential of internalization and accumulation of MNPs in freshwater invertebrates has been mainly shown in bivalvia, gastropoda, and crustacean arthropods. Uptake of the MNPs occurs with different processes known as endocytotic pathways, clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, *etc.* Moreover, the bioavailability of MNPs is influenced by the physicochemical characteristics of MNPs (e.g., size, concentration, and surface chemistry) and the employed experimental conditions (e.g., exposure time and routes).

## 4. Toxicity of MNPs to freshwater invertebrates

MNPs exhibit some unique characteristics compared to their bulk forms, such as a high surface-to-volume ratio and



abundant reactive sites on the surface, which along with their mobility, lead to unexpected environmental hazards. In the last decade, specific physicochemical properties such as size, shape, and surface functionality of MNPs have influenced their toxicity.<sup>16,43,141</sup> Furthermore, various aquatic organisms have been studied to demonstrate MNPs' toxic effects. In this part, we reviewed MNPs' toxicity related studies published after the year 2011 from the Web of Science (<https://www.webofscience.com/wos/woscc/basic-search>) and Google

Scholar (<https://www.scholar.google.com>). Furthermore, we chose three major groups of freshwater organisms within section 3 to discuss and present a compilation of different toxicological measurements for various MNPs in Tables 1–3.

#### 4.1 Bivalvia molluscs

Bivalve molluscs are used as sentinel species for nanotoxicology owing to their high ability for the cellular

**Table 1** Overview of the toxic effects of MNPs on bivalves according to species, and type of MNPs

| Species                       | MNPs                               |              |   | Time | End points <sup>a</sup>  | Ref.     |
|-------------------------------|------------------------------------|--------------|---|------|--|----------|
|                               | Type                               | Size (nm)    | Conc.                                       |      |  |          |
| <i>Bellamyia aeruginosa</i>   | AgNPs                              | 20           | 1, 10, 100 $\mu\text{g g}^{-1}$             | 14 d | ↑GSH (HP, F, gonad, DG), ↑SOD (HP), ↑POD (F), ↓CAT (HP, gonad)   | 24       |
| <i>Coelatura aegyptiaca</i>   | AgNPs                              | 8–19         | 12.5, 25 and 50 $\text{mg L}^{-1}$          | 6 d  | ↑MDA, ↓GSH, ↓CAT, ↑NO concentration  | 145      |
| <i>Corbicula fluminea</i>     | AgNPs                              | 27.66 ± 0.80 | 0.1, 0.5, 2 $\text{mg L}^{-1}$              | 14 d | ↑SOD, ↑CAT, ↑GPx in the mediate concentration<br>↑GSH ↑GST ↓GPx in the highest concentration,<br>↓ammonia excretion and ↓feeding rates   | 160      |
| <i>Elliptio complanata</i>    | AgNPs                              | 80           | 0.8, 4 and 20 $\mu\text{g L}^{-1}$          | 48 h | ↓HSP72 protein, ↑digestive gland lipid peroxidation,<br>↑metallothioneins, ↑DNA strand breaks  | 152      |
| <i>Elliptio complanata</i>    | AgNPs                              | 80           | 0.8, 4 and 20 $\mu\text{g L}^{-1}$          | 48 h | ↑Lipid peroxidation, ↑phagocytosis activity,<br>↓cytotoxicity activity   | 151      |
| <i>Sphaerium corneum</i>      | AgNPs                              | 15           | 5, 25, 50, 100 and 500 $\mu\text{g L}^{-1}$ | 28 d | ↓Reproduction, ↑ROS, ↑CAT, ↓GPx, ↑GST,<br>↓Na <sup>+</sup> /K <sup>+</sup> -ATPase activity  | 37       |
| <i>Ceriodaphnia cornuta</i>   | AgNPs                              | 10–50        | 20, 30, 40 and 50 $\mu\text{g mL}^{-1}$     | 24 h | ↑Mortality and abnormal swimming behavior  | 89       |
| <i>Dreissena bugensis</i>     | AgNPs                              | 70–80        | 10 and 50 $\mu\text{g L}^{-1}$              | 48 h | ↓PK-LDH, ↓F-actin, and ↓protein-ubiquitin (UB)   | 158      |
| <i>Caelatura aegyptiaca</i>   | Ag/SNCs                            | 10–25        | 12.5, 25 and 50 $\text{mg L}^{-1}$          | 6 d  | ↑MDA, ↑NO concentration, ↓GSH, ↓CAT  | 149      |
| <i>Corbicula fluminea</i>     | AuNPs                              | 10           | 0.5, 1.5, 12, 24 ppm                        | 4 h  | ↑Endocytosis gene expression, ↑oxidative stress<br>gene expression, ↑immune system gene expression,<br>↑apoptosis gene expression  | 222      |
| <i>Unio ravoisieri</i>        | Au/TiO <sub>2</sub> NPs            | 10           | 100 and 200 $\mu\text{g L}^{-1}$            | 7 d  | ↓CAT, ↑GST, ↓AChE, ↑H <sub>2</sub> O <sub>2</sub>  | 223      |
| <i>Unio tigridis</i>          | Al <sub>2</sub> O <sub>3</sub> NPs | 40           | 0, 1, 3, 9 $\text{mg L}^{-1}$               | 14 d | ↓SOD, ↓CAT, ↑GST, ↑GPx   | 224      |
| <i>Corbicula fluminea</i>     | CeO <sub>2</sub> NPs               | 20–25        | 10, 100 $\mu\text{g L}^{-1}$                | 6 d  | ↑DNA tail length, ↑Casp-3 activity in the<br>highest concentration   | 153      |
| <i>Dreissena polymorpha</i>   | CeO <sub>2</sub> NPs               | 3–4          | 1 $\text{mg L}^{-1}$                        | 21 d | ↓piGST mRNA expression, ↑hemocyte lysosomal<br>system size, ↓CAT, ↓GST, ↓[LOOH]  | 112      |
| <i>Dreissena polymorpha</i>   | CeO <sub>2</sub> NPs               | 1385 (dH)    | 100 $\mu\text{g L}^{-1}$                    | 14 d | ↑ETS, ↓ROS, ↓SOD, ↓CAT, ↓GPx, ↓GST,  | 147      |
| <i>Dreissena polymorpha</i>   | CeO <sub>2</sub> NPs               | 3 ± 1        | 10 and 100 $\mu\text{g L}^{-1}$             | 4 d  | ↓CAT, ↑GST, ↓haemolymph [Na <sup>+</sup> ]   | 225      |
| <i>Dreissena bugensis</i>     | CuONPs                             | 79 ± 10      | 2, 10 and 50 $\mu\text{g L}^{-1}$           | 96 h | ↑Poly-ubiquitinated protein, ↓LPO, ↓DNA<br>strand breaks, ↓AChE  | 150      |
| <i>Lamellidens marginalis</i> | CuONPs                             | 34 ± 4.5     | 0.5, 1, 5 $\text{mg L}^{-1}$                | 14 d | ↓Hemocyte count, ↓phagocytic efficacy, ↑SOD<br>↓nitric oxide generation, ↓ total protein content<br>in hemocytes, ↓CAT, ↓PhO   | 148      |
| <i>Unio tigridis</i>          | CuONPs                             | 40           | 0, 1, 3, 9 $\text{mg L}^{-1}$               | 14 d | ↓SOD, ↓CAT, ↑GST, ↑GPx   | 224      |
| <i>Limnoperna fortunei</i>    | TiO <sub>2</sub> NPs               | 20           | 1, 5, 10 and 50 $\mu\text{g mL}^{-1}$       | 4 h  | ↓SOD, ↓CAT, ↓protein sulfhydryl content  | 143, 157 |
| <i>Limnoperna fortunei</i>    | TiO <sub>2</sub> NPs               | 21           | 1, 5, 10 and 50 $\mu\text{g mL}^{-1}$       | 4 h  | ↑Tail DNA  | 159      |
| <i>Unio tumidus</i>           | TiO <sub>2</sub> NPs               | <150         | 1.25 $\mu\text{M}$                          | 14 d | ↓ROS, ↓PhO ↑SOD, ↑lipofuscin accumulation,<br>↑TBAR, ↑GSH, ↑GSSG, ↑GSH/GSSG,<br>↑lactate/pyruvate, ↑ALP, ↑cathepsin D total<br>activity, ↑cathepsin D free (outside lysosome)<br>activity, ↓lysosomal membrane stability | 226      |
| <i>Unio tigridis</i>          | TiO <sub>2</sub> NPs               | 21           | 0, 1, 3, 9 $\text{mg L}^{-1}$               | 14 d | ↓SOD, ↓CAT, ↑GST, ↑GPx   | 224      |
| <i>Unio tumidus</i>           | ZnONPs                             | 50–100       | 3.1 $\mu\text{M}$                           | 14 d | ↑Glycogen, ↓glucose, ↓lipids, ↑pyruvate,<br>↓lactate, ↓lactate/pyruvate, ↓ATP  | 162      |

<sup>a</sup> Glutathione (GSH), superoxide dismutase (SOD), POD catalase activity (CAT), malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione-S-transferase (GST), reactive oxygen species (ROS) production, acetylhydrolase (AChE), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), lipid hydroperoxide (LOOH), mitochondrial electron transport system (ETS), lactoperoxidase (LPO), alkaline phosphatase (ALP), phenoloxidase-like (PhO) activity, nitric oxide (NO), adenosine triphosphate (ATP), pyruvate kinase-lactate dehydrogenase (PK-LDH).



Table 2 Overview of the toxic effects of MNPs to gastropods according to species, and type of MNPs

| Species                          | MNPs                                 |                          |   | Time                  | End points  | Ref. |
|----------------------------------|--------------------------------------|--------------------------|---|-----------------------|---|------|
|                                  | Type                                 | Size (nm)                | Conc.   |                       |   |      |
| <i>Bellamya aeruginosa</i>       | AgNPs                                | 20, 40 and 80            | 1, 10 and 100 mg g <sup>-1</sup>  | 14 d                  | ↑Oxidative stress, ↑GSH, ↑SOD, ↑POD, ↑CAT   | 24   |
| <i>Biomphalaria alexandrina</i>  | AgNPs                                | —                        | 3–100 mg mL <sup>-1</sup>   | 24 h                  | Molluscicide, cercaricide, and anti-parasitic effect  | 227  |
| <i>Cipangopaludina chinensis</i> | AgNPs                                | 20–60                    | 20 and 60 mg L <sup>-1</sup>  | 14 d                  | ↑Bioaccumulation on biofilm. NP impacts on ecological receptors and food chains   | 170  |
| <i>Biomphalaria glabrata</i>     | AgNPs                                | 115.17 ± 55.57           | 1.0, 2.5, and 5.0 mg L <sup>-1</sup>  | 30 d                  | ↓Reproduction rate; ↓egg per egg masses, ↓egg masses production per snail   | 106  |
| <i>Lymnaea stagnalis</i>         | AgNPs                                | 10.3 ± 3.4<br>12.8 ± 4.4 | 25 nM L <sup>-1</sup>   | 24 h                  | The presence humic acid ↑uptake AgNPs PVP in contrast with cysteine but did not eliminate uptake of 25 nM L <sup>-1</sup>   | 228  |
| <i>Lymnaea stagnalis</i>         | AgNPs                                | 100                      | 5, 10 and 50 mg L <sup>-1</sup>   | 72 h                  | ↑Memory formation (10 mg L <sup>-1</sup> ). Blocks memory formation (50 mg L <sup>-1</sup> ). Memory recall is context-specific, thus snails trained in AgNPs do not            | 171  |
| <i>Physella acuta</i>            | AgNPs                                | 24–190                   | 0.001, 0.01, 0.1, 1, 10, 100 mg L <sup>-1</sup>                                       | 96 h and 28 d         | ↑Mortality; ↓egg production; ↓snail size at first reproduction, ↑behavior changes   | 176  |
| <i>Potamopyrgus antipodarum</i>  | AgNPs                                | 15                       | 0.10–1000 mg L <sup>-1</sup>  | 28 d                  | ↓Reproduction. AgNPs in low concentrations can modulate 17α-ethynylestradiol activity   | 229  |
| <i>Potamopyrgus antipodarum</i>  | AgNPs                                | 13                       | 100 µg g <sup>-1</sup>  | 2 wk                  | ↓Growth, ↓reproduction  | 230  |
| <i>Racemina luteola</i>          | AgNPs                                | 32.4 ± 2.6               | 4.01, 12.03 and 24.05 mg L <sup>-1</sup>  | 96 h                  | ↑Oxidative stress; ↑CAT; ↓GSH; ↓GST; ↓GPx; ↑MDA; ↑DNA damage  | 231  |
| <i>Biomphalaria alexandrina</i>  | AuNPs                                | —                        | 100–200 mg mL <sup>-1</sup>   | 24 h                  | Modulation and prevention of the infectivity of cercariae and miracidia   | 227  |
| <i>Bellamya aeruginosa</i>       | CuONPs                               | 41.6 ± 4.6               | 180 mg g <sup>-1</sup>  | 28 d                  | ↑Oxidative stress, ↑SOD, ↑CAT, ↑GST, ↑MDA   | 232  |
| <i>Potamopyrgus antipodarum</i>  | CuONPs                               | 6 ± 1                    | 0, 30, 60, 120 and 240 mg g <sup>-1</sup>   | 8 wk                  | ↓Growth rate, ↓feeding rate, ↓reproduction, and ↓bioaccumulation  | 122  |
| <i>Bellamya purificata</i>       | CeO <sub>2</sub> NPs                 | 25                       | 60 mg L <sup>-1</sup>   | 15 d                  | High bioaccumulation factor. No mortality   | 233  |
| <i>Lymnaea stagnalis</i>         | CuONPs                               | 7                        | Db: 4–50 mmol g <sup>-1</sup> and 50–175 nmol g <sup>-1</sup><br>Wb: 4–16 nM to 31 mM | 3–5 h (Db)<br>24 (Wb) | Bioaccumulation associated to toxicity. Toxicity: Db exposures > Wb exposure  | 125  |
| <i>Racemina luteola</i>          | CuONPs                               | 43.5 ± 1.5               | 7 and 21 mg L <sup>-1</sup>   | 5 d                   | ↑Oxidative stress; ↓GSH, ↓GPx, ↓GST. ↑LPO, ↑SOD (lower concentration, 1 d); ↓SOD (5 d). ↓CAT (2 d); ↑CAT (5 d, lower concentration). DNA damage mediated by oxidative stress    | 168  |
| <i>Bellamya aeruginosa</i>       | CuONPs                               | 10                       | 180 µg g <sup>-1</sup>  | 7, 14, and 28 d       | ↑SOD, ↑CAT, and GST↑ (7 d), ↓SOD, ↓CAT, and ↓GST (>14 d)  | 27   |
| <i>Biomphalaria glabrata</i>     | CdTeNPs                              | 3                        | 50, 100, 200, 400 nM  | 24 h                  | Malformations and mortality of embryos and adult snails depending on the concentration. ↑Cytotoxicity (hemocyte apoptosis)  | 234  |
| <i>Biomphalaria glabrata</i>     | γ-Fe <sub>2</sub> O <sub>3</sub> NPs | 5.7                      | 1.0, 10, 100 mg L <sup>-1</sup>   | 10 d<br>28 d          | No effect on fecundity, fertility, mortality of adults, similar hatching rate, no malformation in embryos   | 177  |
| <i>Biomphalaria alexandrina</i>  | SiO <sub>2</sub> NPs                 | 80                       | 50, 100, 200, 400, 600, 800, 1000, 1200 ppm   | 3, 6, 12, 24, 36 h    | Non-embryonated egg masses (1400 ppm/24 h), embryonated pre-hatched one (1450 ppm/12 h).  | 235  |
| <i>Bellamya aeruginosa</i>       | TiO <sub>2</sub> NPs                 | 11.6 ± 2.4               | 5 and 25 mg kg <sup>-1</sup>  | 21 d                  | ↑LPO, ↑PC, ↓Na <sup>+</sup> /K <sup>+</sup> -ATPase, ↑DNA damage  | 236  |
| <i>Cipangopaludina chinensis</i> | TiO <sub>2</sub> NPs                 | 5–10                     | 1818.2 mg L <sup>-1</sup>   | 17 d                  | Bioaccumulation through trophic transfer during plant consumption. ↑Uptake and bioaccumulation  | 237  |
| <i>Cipangopaludina chinensis</i> | TiO <sub>2</sub> NPs                 | 10–20                    | 2 and 6 mg L <sup>-1</sup>  | 14 d                  | Biomagnified through aquatic food chains. NPs show greater movement in the sediment than in the water in a simplified food chain. ↑Bioaccumulation in the semistatic exposition | 238  |
| <i>Racemina luteola</i>          | TiO <sub>2</sub> NPs                 | 34.1 ± 2.7               | 9 and 28 mg mL <sup>-1</sup>  | 7 d                   | ↓GSH, ↓GST, ↑MDA, ↓SOD, ↓CAT (9 mg mL), ↑CAT (28 mg mL).  | 239  |
| <i>Racemina luteola</i>          | TiO <sub>2</sub> NPs                 | 34.1 ± 2.7               | 28, 56, 84 mg mL <sup>-1</sup>  | 96 h                  | ↓GSH, ↓GST, ↑Oxidative stress, ↑MDA   | 172  |
| <i>Racemina luteola</i>          | ZnONPs                               | 22                       | 10, 21 and 32 mg mL <sup>-1</sup>   | 96 h                  | ↓GSH, ↓GST, ↓GPx, ↑MDA, ↑CAT. genotoxicity mediated by oxidative stress   | 166  |
| <i>Biomphalaria alexandrina</i>  | ZnONPs                               | 17.5                     | 25–600 mg mL <sup>-1</sup>  | 24 h<br>21 d          | ↑MDA, ↑NO, ↓GSH, ↓GST, ↓SOD, ↓PTN, ↓Alb, ↑Ch, ↑AST, ↑ALT, ↑ALP, ↑CAT  | 169  |



**Table 3** Overview of the toxic effects of MNPs to crustacean according to species, and type of MNPs

| Species                      | MNPs   |   |   | Time  | End points  | Ref. |
|------------------------------|--|---|---|-------|---|------|
|                              | Type   | Size (nm)                                     | Conc.   |       |   |      |
| <i>Daphnia magna</i>         | AgNPs  | 18.2 ± 10.1                                   | 0.5, 1, 3, 5, 10 µg L <sup>-1</sup>                                     | 48 h  | ↑AChE, ↓ROS, ↑GSH, ↑CAT   | 178  |
| <i>Daphnia magna</i>         | AgNPs  | 6.3–8.4                                       | 50, 100, 200, 300 µg L <sup>-1</sup>                                    | 21 d  | ↑Mortality (dose–effect)  | 240  |
| <i>Gammarus fossarum</i>     | AgNPs  | 20, 23 and 27                                 | 1, 3 µg L <sup>-1</sup>   | 72 h  | ↓Haemolymph osmolality, no significant in antioxidant responses, defense mechanisms, cellular damage, energy reserves and ventilatory activity  | 180  |
| <i>Daphnia magna</i>         | AgNPs  | 40 and 110                                    | 2 µg L <sup>-1</sup>  | 24 h  | Citrate-coated AgNPs were more toxic than PVP-coated AgNPs, and 40 nm AgNPs were more toxic than 110 nm AgNPs   | 76   |
| <i>Gammarus fossarum</i>     | AgNPs  | 40  | 0, 0.5, 5 µg L <sup>-1</sup>  | 15 d  | ↑Catalase and chitinase gene expression, ↑digestive lysosomal system, ↓locomotor activity   | 23   |
| <i>Gammarus fossarum</i>     | AgNPs  | 20, 40 and 80                                 | 1, 2, 4, 8, 10 µg L <sup>-1</sup>                                       | 72 h  | ↑CuZnSOD gene expression  | 241  |
| <i>Daphnia lumholtzi</i>     | AgNPs  | 9.8 ± 0.8                                     | 0.1, 0.5, 1, 2, 5 µg L <sup>-1</sup>                                    | 21 d  | ↑Time to first brood, ↓number of offspring per female, ↓survival  | 183  |
| <i>Daphnia lumholtzi</i>     | AgNPs  | 9.8 ± 0.8                                     | 0.2, 0.5 µg L <sup>-1</sup>   | 21 d  | ↓Reproduction rate  | 242  |
| <i>Ceriodaphnia cornuta</i>  | AgNPs  | —   | 4, 5, 10, 15 and 20 µg ml <sup>-1</sup>                                 | 24 h  | ↑Mortality rate, ↑DNA damage  | 243  |
| <i>Moina macrocopa</i>       | AgNPs  | 20 and 40                                     | 0.011 and 0.022 mg L <sup>-1</sup>                                      | 48 h  | ↓AChE, ↓SOD, ↑CAT, ↑GST, ↓trypsin activity, ↓β-galactosidase activity, ↑phosphatase activity  | 179  |
| <i>Paratya australiensis</i> | AgNPs  | 10.56 ± 2.27, 9.27 ± 1.29, 13.68 ± 0.76       | 30 µg L <sup>-1</sup>   | 28 d  | ↑TBARS, ↑CAT  | 138  |
| <i>Ceriodaphnia cornuta</i>  | AgNPs  | 23 ± 2  | 10, 20 40 and 50 µg L <sup>-1</sup>                                     | 24 h  | ↑Mortality, abnormal swimming, ↓heart rate, ↓thoracic limb movement   | 186  |
| <i>Daphnia magna</i>         | AgNPs  | 65  | 3.5, 8.1, 0.43, 1.05 µg L <sup>-1</sup>                                 | 24 h  | ↓Sensory development, damage repair genes   | 244  |
| <i>Daphnia magna</i>         | AgNPs  | 5–50  | 10, 20, 30, 40 and 50 µg L <sup>-1</sup>                                | 48 h  | ↓Survival   | 233  |
| <i>Cypridopsis vidua</i>     | AgNPs  | —   | 10, 50, 150, 250, 350, 450, 550 and 1000 mg L <sup>-1</sup>             | 48 h  | ↑Immobilization   | 245  |
| <i>Ceriodaphnia cornuta</i>  | Sn-AgNPs   | 10–50   | 1, 2, 5, 10, 20, 30, 40 and 50 µg ml <sup>-1</sup>                      | 24 h  | ↑Mortality, abnormal swimming behaviour   | 89   |
| <i>Daphnia magna</i>         | Al <sub>2</sub> O <sub>3</sub> NP                        | <50   | 3.12, 6.25, 12.5 and 25 mg L <sup>-1</sup>                              | 21 d  | ↓Survivors, ↓body length, ↓age at first brood, ↓neonates per surviving adult, ↑ROS, ↑CAT, ↓SOD, ↓GSH, ↑MDA, ↓average swimming distance of neonates (48 h)   | 184  |
| <i>Daphnia magna</i>         | CeO <sub>2</sub> NPs                                     | 5   | 10 and 100 µg L <sup>-1</sup>   | 48 h  | ↓CAT (ceria@chitosan type), ↓GST (ceria@alginate type), ↑ROS (ceria@alginate type), ↑swimming activity, ↑swimming velocity (ceria@alginate type)  | 182  |
| <i>Daphnia sp.</i>           | CuONPs   | 45 ± 3  | 0.1, 1, 5, 10 and 25 mg L <sup>-1</sup>                                 | 24 h  | ↓Number of motile counts  | 246  |
| <i>Daphnia magna</i>         | CuONPs   | <50   | 0.5, 1, 1.5, 2, 2.5 and 3 mg L <sup>-1</sup>                            | 120 h | ↓Survival   | 247  |
| <i>Daphnia magna</i>         | CuONPs   | <50   | 0.07 and 15 mg L <sup>-1</sup>  | 14 d  | ↑Mortality (Wb), ↓average number of neonates produced per adult at high concentration (Fb), ↓total number of broods produced per adult (Wb) at the high concentration, ↓total number of broods produced per adult (Fb) at the low concentration | 126  |
| <i>Daphnia magna</i>         | CuONPs   | 110.34 ± 56.58, 38.27 ± 23.05                 | 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 5, 25, 50, 75 and 100 mg L <sup>-1</sup> | 48 h  | ↓Survival   | 248  |
| <i>Daphnia magna</i>         | SiO <sub>2</sub> NPs, Fe <sub>3</sub> O <sub>4</sub> NPs | 20–30, <20                                    | 5, 50, 100, 250, and 250 mg L <sup>-1</sup>                             | 96 h  | ↑Mortality rate   | 249  |
| <i>Daphnia magna</i>         | TiO <sub>2</sub> NPs                                     | 29 ± 8  | 1, 10, 100 ppm  | 48 h  | ↑Mortality, ↑swimming distance  | 187  |
| <i>Daphnia magna</i>         | TiO <sub>2</sub> NPs                                     | <25   | 0.1 and 1 mg L <sup>-1</sup>  | 2 d   | No significant ROS increase and MT induction.   | 250  |
| <i>Ceriodaphnia dubia</i>    | TiO <sub>2</sub> NPs                                     | 9.5 ± 1 (anatase), 26 ± 3 (rod-shaped rutile) | 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75 toxic unit                      | 48 h  | ↑Mortality rate   | 251  |
| <i>Daphnia similis</i>       | TiO <sub>2</sub> NPs                                     | <25   | 1 and 10 mg L <sup>-1</sup>   | 96 h  | ↓Growth rate  | 181  |



Table 3 (continued)

| Species                | MNPs                 |           |  | Time | End points   | Ref. |
|------------------------|----------------------|-----------|--|------|--|------|
|                        | Type                 | Size (nm) | Conc.  |      |  |      |
| <i>Daphnia similis</i> | TiO <sub>2</sub> NPs | <25       | 7, 75, and 750 mg L <sup>-1</sup>                    | 24 h | ↓CAT, ↓AP, ↓SOD  | 181  |
| <i>Daphnia magna</i>   | ZnONPs               | 20–40     | 0.009, 0.014, 0.027, 0.058, 0.131 mg L <sup>-1</sup> | 21 d | ↓Average brood size, ↑time to first brood, ↓broods per female  | 252  |
| <i>Daphnia pulex</i>   | ZnONPs               | 61 ± 12   | 0.06 mg L <sup>-1</sup>                              | 24 h | ↓Na <sup>+</sup> /K <sup>+</sup> ATPase, ↓RNA-binding protein, ↓rRNA methyltransferase, ↓signal recognition particle receptor, ↓signal peptidase | 253  |
| <i>Daphnia magna</i>   | ZnONPs               | 10–30     | 0.2, 1, 5, 10, 25, 50 ppm                            | 72 h | ↓Alive account   | 103  |
| <i>Daphnia magna</i>   | ZnONPs               | <50       | 0.1 and 0.3 mg L <sup>-1</sup>                       | 21 d | ↓Survival probability  | 254  |
| <i>Daphnia magna</i>   | ZnONPs               | 63 ± 11   | 0.1 mg L <sup>-1</sup>                               | 14 d | ↓Survival, ↓body length and embryo numbers of the first brood, ↓SOD, ↓GST, ↓CAT, ↓MDA  | 124  |

internalization of MNPs. Table 1 describes the different bivalve species used in nanotoxicology studies and their toxic responses. Most articles reported the concentration effect in freshwater bivalves, focusing on short-term toxicity with an exposure time between 2 h and 14 d. Numerous studies indicate that MNPs impose bivalves' toxicity mainly through the functional parameters, such as immunotoxicity, oxidative stress, DNA damage, lysosomal damage in bivalve tissues, and protein expression changes (Table 1). In particular, the bivalve immune system represents a significant target for MNPs.<sup>110</sup> Biochemical parameters are widely used to monitor the physiology of aquatic species to assess the impact of MNPs as early-warnings biomarkers. In invertebrates, parameters involved in antioxidant defenses (*e.g.*, glutathione peroxidase, GPx; superoxide dismutase, SOD; catalase, CAT), oxidative stress (*e.g.*, lipid peroxidation, LPO), and detoxification (*e.g.*, glutathione S-transferases, GST) are commonly analyzed.<sup>142</sup> In the literature, hepatopancreas, digestive gland, gonad, and hemocyte cells are the frequently tested organs to assess the effects of MNPs (*e.g.*, AgNPs, CeO<sub>2</sub>NPs, CuONPs, and TiO<sub>2</sub>NPs).

Currently, reactive oxygen species (ROS) and free radical production may account for the mechanism of cytotoxic effects exerted by MNPs in bivalves.<sup>143,144</sup> The MNPs may release extracellular metal ions, which penetrate the cell and induce oxidative stress by free radicals or ROS production and/or metallothionein (MT) induction. The oxidative stress induced by MNPs mainly includes the disruption of the antioxidant defense system (SOD, CAT, GPx, GST),<sup>24,37,145–148</sup> LPO,<sup>149–152</sup> increased protein modification (*e.g.*, ubiquitination),<sup>150</sup> and DNA damage (DNA strand breaks).<sup>150,152,153</sup> Oxidative damage induced by MNPs in bivalves depends on the size, composition, concentration, and exposure time (Table 1). The MNP's size is the dominant factor determining the oxidative stress change and is associated with its high surface area. For example, 80 nm AgNPs induced greater MT levels, LPO, and DNA strand breaks in the digestive gland of freshwater mussel *Elliptio complanata*, compared to 20 nm AgNPs.<sup>152</sup> The above result suggests a more critical release of dissolved Ag from larger

AgNPs. However, the relationship between the hydrodynamic diameter and morphology of MNP aggregates and oxidative stress in bivalves has not been well-established. Notably, previous studies also showed that smaller sizes could enter cells *via* endocytosis more easily than larger sizes,<sup>76,154</sup> and might generate severe effects on organisms.<sup>76,155</sup> Oxidative stress induced by MNPs also depends on types of tissues and cells. For example, the gills of *C. aegyptiaca* are more susceptible to oxidative stress induced by AgNPs than the hepatopancreas.<sup>145</sup>

The immune system of bivalves is a sensitive target of MNP toxicity. Hemocytes are the most investigated cell type among the analyzed species (Table 1). Generally, upon exposure and crossing the epithelium of digestive gland tubules, MNPs can translocate from the digestive system into the circulatory hemocytes.<sup>143</sup> Furthermore, the endocytic and lysosomal pathways are the major subcellular fate of MNPs in bivalve species.<sup>156</sup> For example, TiO<sub>2</sub>NPs have been demonstrated to internalize into the hemocytes of the golden mussel *Limnoperna fortunei*, which can penetrate and be phagocytosed by hemolymph cells, being able to damage the hemocyte membrane.<sup>157</sup> The TiO<sub>2</sub>NPs can induce a redox imbalance in mussel cells, decrease SOD and CAT activities, and induce protein sulfhydryl content decrease after TiO<sub>2</sub>NP exposure.<sup>157</sup> MNPs induce ROS production that leads to changes in the immune system due to inflammatory processes (reduction in phagocytic activity and hemocyte viability). Changes in phagocytosis activity, cell viability/density, stimulation of lysosomal enzyme release, ROS production, mitochondrial damage, and DNA damage were observed in bivalve hemocytes after exposure to different ENMs, such as AgNPs, CuNPs, and TiO<sub>2</sub>NPs.<sup>37,148,158,159</sup>

Behavioural biomarkers, such as the feeding rate and valve opening, are essential tools to assess the MNPs' toxicity in bivalves. As exposure concentrations of AgNPs are elevated, the ammonia excretion and feeding rates of *C. fluminea* diminished initially and then increased.<sup>160</sup> This tendency indicated that lower concentration exposures (0.1 and 0.5 mg L<sup>-1</sup>) induced ROS accumulation in the body, which resulted in a certain degree of oxidative damage in cells. In contrast,



the organisms' antioxidant enzyme defense system had not yet been activated. When the concentration of AgNPs increased to  $2 \text{ mg L}^{-1}$ , the antioxidant enzyme defense system produced many enzymes to eliminate ROS, protecting the body from oxidative damage and increasing feeding and excretion capacity.<sup>160</sup> Bioenergetic-related traits provide essential advantages for environmental stress assessment as they permit integration of the physiological effects of environmental stressors with different mechanisms of action and provide a direct link between the physiological change and the organism's fitness.<sup>161</sup> ZnONP exposure (14 d) significantly decreased the glycogen, glucose, and lipids of *U. tumidus*.<sup>162</sup> Meanwhile no damage to proteins and lipids was found in marine clam *Ruditapes philippinarum* subjected to environmentally relevant ZnONP concentrations for a seven day exposure period.<sup>163</sup> The species difference may reflect a short exposure time insufficient to trigger a stress response in the latter study. It also emphasizes the need for more investigations of long-term exposures to MNPs to assess their biological and toxic impacts.

#### 4.2 Gastropod molluscs

Toxic effects in gastropods induced by MNPs depended on the size, aggregation capacity, target cell and tissue. In general, small MNPs have a high surface area and dissolution potential, enter the cell membrane, and cause oxidative stress.<sup>76</sup> On the other hand, small particles may also tend to aggregate or be more readily absorbed by organic matter or the sediment present in the environment.<sup>42</sup> For example, the toxicity of larger AgNPs (40 and 80 nm) to *B. aeruginosa* was higher than small AgNPs (20 nm) after a 14 d exposure period.<sup>24</sup> The small CeO<sub>2</sub>-NPs (3.8 vs. 185 nm) induced a high accumulation rate and trophic transfer potential since small MNPs were more bioavailable in the water column.<sup>164</sup> In contrast, the larger MNPs remained associated with the sediment.

The primary mechanism of action and toxicity of MNPs to gastropods is mainly associated with the oxidative stress-related mechanism.<sup>16</sup> As we reviewed in Table 2, oxidative stress is the primary effect in gastropods among the toxicity induced by MNPs. MNPs led to an imbalance between ROS production and the capacity of the antioxidant defense system (SOD, CAT, GPx, and GST), and increased LPO of different tissues (e.g., hepatopancreas, digestive gland, mantle) and hemolymph cells<sup>24,165–167</sup> (Table 2). Furthermore, MNPs can also induce protein carbonylation (PC),<sup>167</sup> increase the total lipids and cholesterol levels as well as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP),<sup>167</sup> inhibit the Na<sup>+</sup>/K<sup>+</sup> ATPase,<sup>27</sup> and induce DNA damage.<sup>165,166,168,169</sup> The oxidative damage induced by MNP exposure in gastropods also was concentration and exposure time-dependent, which has been demonstrated for AgNPs, CuONPs, TiO<sub>2</sub>NPs, and ZnONPs.<sup>24,27,170,171</sup> Generally, prolonged exposure and higher concentration of MNPs induced more significant oxidative damage. For example, short-term exposure (7 d) to CuO-NPs led to oxidative stress of freshwater snail *B.*

*aeruginosa*. In comparison, long-term exposure (>14 d) led to oxidative damage, which means prolonged exposure will enhance the MNPs' ecotoxicity risk to organisms.<sup>27</sup> Moreover, exposure under high concentration ( $21 \mu\text{g L}^{-1}$ ) induced more significant oxidative stress of freshwater snail *Lymnaea luteola* than  $7 \mu\text{g L}^{-1}$ .<sup>172</sup> Cell and tissue-specific responses to oxidative damage induced by MNPs were reported for snails. Among the most studied organs concerning oxidative stress induced by MNPs is the digestive gland, possibly due to its higher accumulation capacity and role in metal detoxification. The digestive gland of *B. aeruginosa* exposed to AgNPs was more susceptible to oxidative stress than gonads, visceral mass, and foot/muscle.<sup>24</sup> A similar tissue-specific response also was observed in *B. aeruginosa* exposed to CuONPs.<sup>167</sup> The hemocytes are immune cells representing the first line from external stressors by rapidly initiating the immune response. Hemocytes are also well-known as the most studied cells to assess the effect of oxidative stress caused by MNPs. The hemocytes of *B. Alexandrine* demonstrated oxidative stress after ZnONPs, while the visceral mass response was observed only at the highest level.

Another possible toxicity mechanism of MNPs is linked to the release of metal ions in freshwater. However, the Croteau group predicted that around 80% of the bioaccumulation of Cit-AgNPs by *L. stagnalis* was driven by uptake of particulate Ag.<sup>173</sup> The freshwater snails *L. stagnalis* and *Physa acuta* exposed to waterborne Ag showed comparable uptake rate constants for Ag<sup>+</sup> and AgNPs.<sup>128,174</sup> However, notably, the Ag<sup>+</sup> elimination rate was not as high as the AgNP form, suggesting that the ion form may have more time to trigger the stress.<sup>174</sup> Unlike the endocytosis pathway for MNPs, the uptake mechanism of the metal ion is mainly *via* ion transport channels, such as the proton-coupled Na<sup>+</sup> channels.<sup>127</sup> The uptake pathway is a vitally important factor in determining the intracellular fate and toxicity of the AgNPs in the estuarine mud snail *Peringia ulvae*.<sup>120</sup> MNPs usually are endocytosed by the clathrin-mediated pathway, which directs towards lysosomal degradation.<sup>120</sup> Meanwhile the conclusive demonstrations of intracellular fates of metal ions remain elusive.

Fecundity has been suggested to be the most sensitive endpoint to assess the likely effects of contaminant exposure in freshwater organisms. For egg-clutches per snail *Biomphalaria glabrata*, there was significant inhibition after AgNP exposure.<sup>175</sup> Similar low egg production was observed in the snail *Physa acuta* under AgNP exposure.<sup>176</sup> In *P. antipodarum*, after nine-week CuONP exposure, ~70% of the snails stopped their reproduction.<sup>118</sup> On the other hand, the hatching success of the snail *P. acuta* was more sensitive to silver in the ionic form than the AgNP exposure.<sup>175</sup> The egg masses have mucous components, and compared with AgNPs, Ag<sup>+</sup> may penetrate through the egg mass membrane more easily, while AgNPs may be embedded on the egg mass surface which lowered their penetration.<sup>106</sup> However,  $\gamma\text{-Fe}_2\text{O}_3$  NPs generate no effects on the fecundity, hatching rate and mortality of *B. glabrata*, and no malformation in embryos.<sup>177</sup>



### 4.3 Crustacean arthropods

AgNPs and *Daphnia* spp. were the most studied MNPs and crustacean arthropod species, as summarized in Table 3. At present, most of the results have been analysed following the OECD guidelines for the short-term *in vitro* assay method. Primary outcome measures include mortality, time to first brood, number of offspring per female, reproduction rate, and swimming behaviours (Table 3). Biomarkers for early-warning purposes are also extensively used (Table 3).

One of the most widely accepted toxicity mechanisms proposed for AgNPs is the generation of ROS, which can lead to lipid peroxidation inducing, finally, oxidative stress.<sup>178</sup> Implementation of biochemical biomarkers (e.g. SOD, CAT and GST) is considered a promising tool for ecotoxicological applications as early warning indicators, which play a significant role in relieving ROS-associated stress. AgNPs enhanced the SOD, CAT and GST activity of *Moina macrocopa*.<sup>179</sup> Similar results were also found in *D. magna* under AgNP exposure.<sup>178</sup> Meanwhile no significant alterations in antioxidant responses, defense mechanisms and cellular damage were detected in *G. fossarum*.<sup>180</sup> In addition, many other opposite results have also been reported.<sup>124,181,182</sup> An apparent increase in AChE activity in *D. magna* and *M. macrocopa* was observed upon exposure to AgNPs.<sup>178,179</sup> However, CeO<sub>2</sub> NP exposure did not affect the AChE activity in *D. magna*.<sup>182</sup> Whether the alteration of antioxidative stress enzymes affects freshwater invertebrates' cholinergic system needs further research.

AgNPs enter into an aquatic system mainly through ingestion. The overall growth is closely associated with its digestive capacity to break food into small absorbable molecules in the digestive tract, and digestive enzymes perform this task. For example, AgNPs inhibited the digestive enzymes (trypsin, amylase and  $\beta$ -galactosidase) of *M. macrocopa*. Moreover, AgNPs of 40 nm cause more hazards to the digestive enzyme than NPs of 20 nm. This is likely due to the rapid clearance of smaller AgNPs from the digestive system compared to the large ones.<sup>179</sup> Experiments examining chronic stress and its impacts on *Daphnia* spp. have focused on growth and reproduction. MNP exposure can lead to short body length, delayed breeding time, decreased offspring, etc.<sup>126,183,184</sup> The MNPs can physically damage *D. magna* by adhering to surfaces such as tentacles, skin, and cases through physical adsorption. For example, TiO<sub>2</sub> NPs have also been attached to the *D. magna* shell, forming a stable 'shell layer' that affects the molting process.<sup>36</sup>

Moreover, after 21 d of exposure, the longevity, growth, and reproduction of *D. magna* decreased significantly with increasing Al<sub>2</sub>O<sub>3</sub> NP concentration from 6.25 to 25 mg L<sup>-1</sup>.<sup>184</sup> The survival, growth and reproduction of *Daphnia lumholzi* were also reduced after AgNP exposure from 0.1 to 5  $\mu$ g L<sup>-1</sup> for 21 d.<sup>183</sup> Considering that dietary exposure may pose different effects than direct routes, the toxic effects of CuONPs on *D. magna* were evaluated for two chronic exposure scenarios, i.e., indirect feeding and direct

waterborne exposure.<sup>126</sup> The results evidenced that the total number of broods produced per adult decreased in the direct exposure and feeding direction. This indicates that CuONP exposures could impact the reproduction of *D. magna* regardless of the exposure scenario.

The swimming behaviour of *Daphnia* could be an excellent biomarker in toxicity assessment.<sup>185</sup> Decreased swimming activity, shorter cumulative distance and loss of trajectory orientation of swimming were observed in *D. magna*.<sup>89,182,184,186,187</sup> Such behaviour damage could be related to the interactions of MNPs with the carapace of the daphnids and perhaps related to the high energy demand and nervous system disorders.<sup>185</sup> The decreased swimming capacity after exposure to MNPs may indicate that the daphnids are directing energy to conserve processes essential to neutralize the toxicity of the MNPs, leading to reduced feeding and a decrease in growth and reproduction.<sup>185</sup>

In summary, the toxic effects of MNPs on freshwater invertebrates are influenced by the type, size, concentration, morphology of the MNPs and exposure time. The mechanisms of toxicity are currently characterized by ion release and the small size effects of MNPs. In addition, it is also related to the physiological properties of the test organisms themselves, as different species could pose different tolerances to nanomaterials, which induce different organismal defense systems. The overview of MNP bioaccumulation with three major freshwater organisms induced by environmental transformation and the mechanisms of cell damage by MNPs are illustrated in Fig. 2.

Remarkably, when assessing the deleterious effects MNPs, the above results are more based on laboratory simulations.<sup>18,104,158,182</sup> However, complex environmental factors (e.g., pH, NOM, IS, ligands) in natural waters make the toxicity results more complicated than the laboratory culture medium. Very few researchers have addressed the comparison of toxic effects of MNPs in different test media. One recent study investigated the toxicity of AgNPs to *D. magna* in culture medium (M4) and surface water.<sup>188</sup> They found that AgNPs exhibited lower toxicity in surface water than in M4 medium, showing a higher median effect concentration (EC50) within 48 h. Furthermore, a higher survival rate was observed in the surface water medium after 21-day exposure.

As mentioned, NOM may contribute to the aggregation of MNPs *via* coating or binding, and di- and multi-valent cations in natural water may also enhance the aggregation.<sup>42,60</sup> Moreover, NOM exhibited strong Ag<sup>+</sup>-binding capabilities, protecting the body from injuries caused by AgNPs and free Ag<sup>+</sup>.<sup>189,190</sup> Similarly, TiO<sub>2</sub>NPs and AgNPs in the standard culture medium displayed more severe toxic effects (e.g., impaired growth, higher mortality, and lower reproductive success) on *D. magna* than those in synthetic European Class V lowland water.<sup>191</sup> The above results indicate that a suitable medium plays an important role when accessing the potential hazards of MNPs. Based on limited information, many presently available research



studies may underestimate the truth, and additional work is required to understand possible mechanisms of toxicity in real exposure scenarios.

In toxicology experiments, exposure concentration is critical to evaluate physiological endpoints. A recent review summarizes the measured environmental concentrations (given by  $\text{ng L}^{-1}$ ) of several common MNPs.<sup>192</sup> It could be argued that the exposure concentrations used in most of the existing studies are too high to be of physiological significance. Notably, given that MNP concentration in natural environments is forecast to increase drastically by the next century,<sup>193,194</sup> MNP pollution may become a major threat to organisms in the future. Thus, more attention is required. High concentration exposure provides implications on further environmental change, and the environmental-related concentration scenarios can inform us of current MNP pollution governance. Recent technological breakthroughs in single-particle ICP-MS (SP-ICP-MS) have allowed the analysis of size as small as 4.9 nm for AgNPs and concentration as low as 27 particles per  $\mu\text{L}$ .<sup>195</sup> Without technical limitations, environmental-related exposure is encouraged to be conducted to better reflect more plausible results.

## 5. Case study: understanding the effect of AgNP transformation on the toxicity in freshwater invertebrates by meta-analysis

As mentioned above, studies on the toxic effects of MNPs clarified that ecotoxicity is dependent on the intrinsic properties of MNPs, the composition of the environmental media, and the experimental ways (waterborne and dietborne). Results of the different assays of toxic responses to MNPs are sometimes controversial. It is worth investigating the causes of inconsistent results from experiments. The meta-analysis includes independent results, is an approach to explore a correlation between target variables and toxic responses, and identifies the main variables that potentially contribute to heterogeneity in conclusions. We selected studies in 2011 to include in our meta-analyses *via* Google scholar, using the term “Freshwater invertebrate AND Nanoparticle OR Bivalve OR Gastropoda OR Crustacean OR viability OR reproduction OR metabolic stress OR physicochemical barriers OR Immunocytes OR Stem cells OR Protein corona OR Cytokine-like protein OR Omic.” This initial search yielded 1320 papers, 260 duplicates, and 621 citations after the title and abstract screen were excluded. 1060 full texts were assessed, and 84 articles were identified to be related to quantitative experimental research on MNPs (Fig. 3). Of these, AgNPs are of particular interest in the MNP-related toxicological profile (Fig. 4). On top of AgNPs,  $\text{TiO}_2$  NPs, ZnONPs, and CuO NPs also attract considerable attention. Based on these research studies, we chose AgNPs as a case study and finally, 14 articles were included for

meta-analysis. Oxidative stress is convenient in measuring ecotoxicity because cells respond to oxidative stress by exerting several protective responses measured by enzymatic or genetic expression responses.<sup>196</sup>

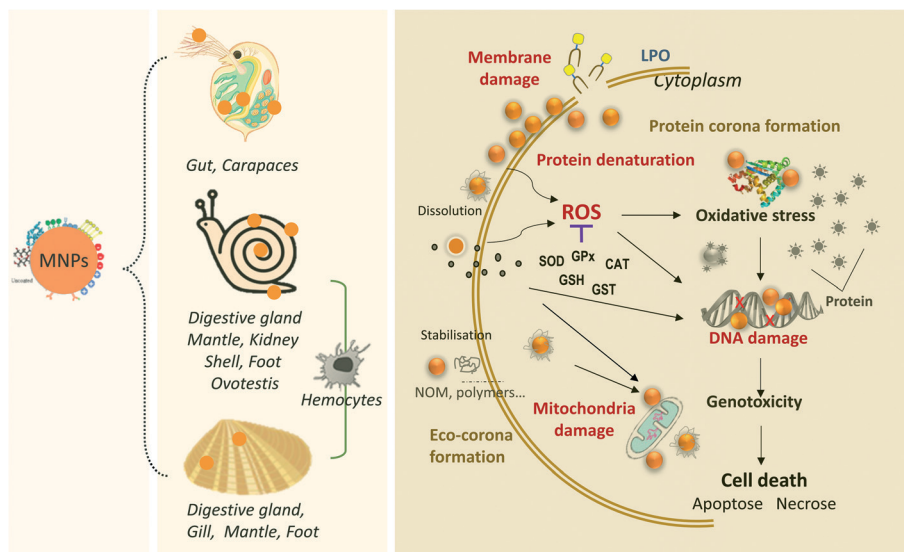
During data assessment, when the original data from the experiment could not be referred to in the article, the numerical values were extracted by reading the graphs with a digital ruler (GetData Graph Digitizer). We extracted the following information from each study: publication characteristics (title of the study, first author and publication year), data on the experimental and control groups ( $n$ , mean, SD), the species, type, size, and concentration of NPs, and exposure period. A complete list of information extracted is included in Table S1†

A random-effects model was chosen for the meta-analysis. Continuous variables were estimated as standardized mean differences (SMDs) with 95% confidence intervals (95% CI) between the experimental and control groups. The absolute effect sizes (SMDs) was interpreted as follows:  $\text{SMD} < 0.2 =$  “negligible”,  $\text{SMD} 0.2$  to  $0.5 =$  “small”,  $\text{SMD} 0.5$  to  $0.8 =$  “medium”, and  $\text{SMD} > 0.8 =$  “large”, as per Cohen’s classification.<sup>197</sup> The heterogeneity between studies was assessed using the Chi-squared test, with inconsistency index ( $I^2$ )  $> 50\%$  and  $p < 0.1$  considered significant heterogeneity.<sup>198</sup> Meta-regression analyses were also conducted to identify independent sources of between-study heterogeneity. Then subgroup analyses were performed in terms of MNP size, concentration and exposure time. Funnel plots were used to investigate the existence of publication bias in studies with a total of more than ten included data. All analyses were implemented in Stata MP 16.0 (Stata Corp., College Station, Texas, TX, USA, 2017).

Analyses showed that ROS, SOD, CAT, GSH, LPO and AChE levels were higher in the experimental group than in the control group with medium effect sizes ( $\text{SMD} 0.52\text{--}3.99$ ,  $p = 0\text{--}0.008$ ,  $I^2 = 41.3\text{--}93.7\%$ ). In contrast, GST and GPx were lower than controls also with large effect sizes ( $\text{SMD} -1.12\text{--}-1.47$ ,  $p < 0.001$ ,  $I^2 = 85.6\text{--}86.5\%$ ) (Table S2†). Based on the significant statistical heterogeneity observed for almost all the biomarkers ( $I^2 > 50\%$ ), the meta aggression results identified a specific heterogeneity source for each biomarker (Table S1†). Among the various potential covariates, the size, coating of AgNPs, exposure concentration, and time were associated with study heterogeneity (Table S1†). The subgroup analysis explored the source of heterogeneity by the particle size of AgNPs ( $<30$  nm and  $>30$  nm), coating (CIT, PVP, tyrosine and bared), concentration ( $<30 \mu\text{g L}^{-1}$  and  $\geq 30 \mu\text{g L}^{-1}$ ), and exposure time ( $<14$  d and  $14$  d) (Table S2†). The SMD values of ROS and CAT indicated that they had been induced more by AgNPs with  $>30$  nm than smaller sizes ( $p < 0.01$ , Fig. S1a and c†). It was worth noting here that the SMD value for ROS is as high as 16.86.

In terms of coating, PVP and tyrosine decreased the AChE activity with large SMD values ( $-8.34$  and  $-8.68$ , respectively), while Cit and bare enhanced AChE also with large SMD values ( $0.8$  and  $1.72$ , respectively) (Fig. S1h†). According to



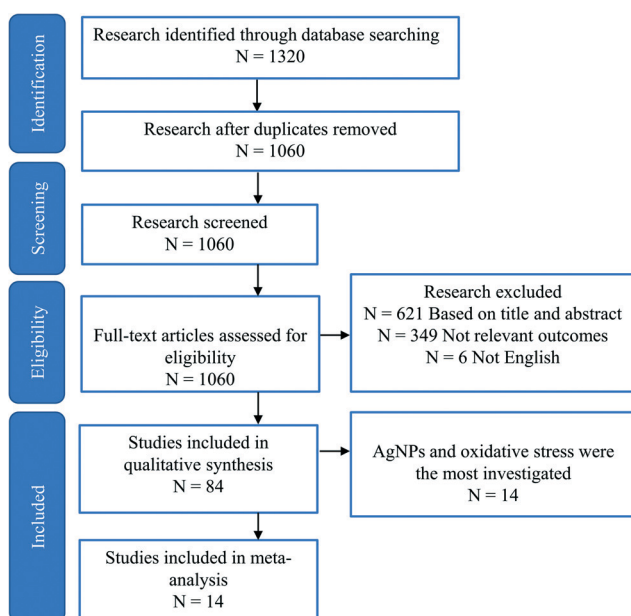


**Fig. 2** Overview of MNP bioaccumulation with three major species of freshwater organisms and the mechanisms of cell damage by MNPs (damage of membranes, DNA and mitochondria; lysosome dysfunction, generation of reactive oxygen species, ROS, disturbance of protein functions).

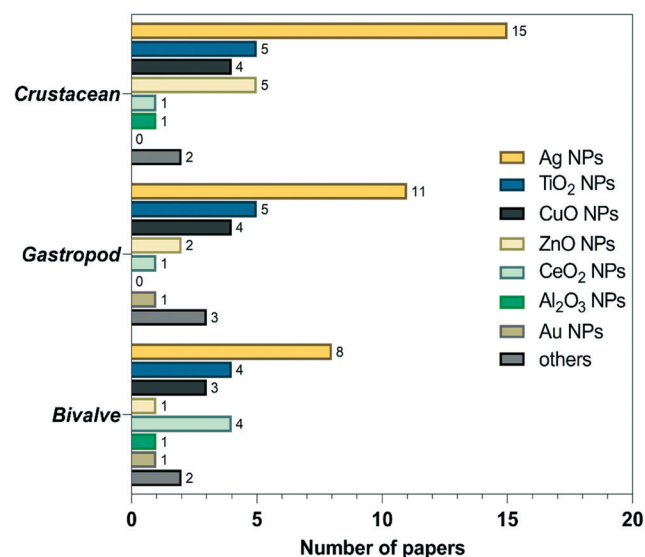
our results, lower exposure concentration ( $<30 \mu\text{g L}^{-1}$ ) triggered higher toxicity in GST ( $p < 0.01$ , Fig. S1d†), while higher concentration ( $\geq 30 \mu\text{g L}^{-1}$ ) had higher activity for inducing a rise in the LPO ( $p < 0.01$ , Fig. S1g†) and decrease in GPx ( $p < 0.01$ , Fig. S1f†). Our result showed that SOD and GSH in freshwater invertebrates are mainly affected by the exposure duration, in which acute exposure time ( $<14$  d) produces a more drastic decrease in SOD activity ( $p = 0.003$ , Fig. S1b†) and GSH activity ( $p < 0.01$ , Fig. S1e†).

Lipid peroxidation damage marks the oxidative stress endpoint, which was observed when the exposure

concentration was higher than  $30 \mu\text{g L}^{-1}$ . The absent toxicity of lower exposure concentration could be reasoned with the high tolerability of organisms. On the other hand, a short exposure time in these studies might also be a part of the explanations. However, the occurrence of toxic effects is a complex process influenced by many factors. Ideally, prolonging the exposure could exert higher toxicity with increased probability of organism-MNPs contact. However, these results could also be overestimated, and the accumulation and elimination process could co-occur with increasing concentration. For example, a recent study reported the elimination rates of *D. magna* as the ZnONP concentration increased.<sup>103</sup> However acute responses are



**Fig. 3** Flow chart of the study selection process.



**Fig. 4** MNP research areas sorted by the number of articles published.



usually determined simultaneously and thus do not track the cumulative exposures over time.<sup>199</sup> This evaluation enables the prioritization of providing information for pollution risk assessment. Indeed, limited information is available from such data without considering the potential chronic effects. Thus, short time and prolonged exposure for months or even an entire life cycle is needed to characterise acute and chronic toxicity.

ROS generation is an appropriate parameter considered as one of the potential mechanisms for MNP toxicity in freshwater organisms. In our meta-analysis, size and time are the most influential drivers affecting ROS production (Table S1†). In terms of physical properties, the decrease in the particle size of MNPs usually leads to changes in the crystalline surface structure, increase in the specific surface area, and curvature. In terms of chemical properties, the decrease in particle size of MNPs increases the density of surface functional groups, surface energy, and surface charge density. The surface energy and surface charge density also increase.<sup>200</sup> These changes lead to the excess energy and surface activity of MNPs, leading to interfacial reactions. It was found that the pore sizes of biological membranes such as cell membranes (0.4–1 nm) and nuclear membranes (50–70 nm) are mainly in the nanometer range,<sup>201</sup> and the diameter of cell wall pores is also in the range of 5–20 nm.<sup>202</sup> Theoretically, when the particle size of MNPs is smaller than the size of cell wall pores and biofilm micropores, MNPs can directly enter the cell and even the organelle.<sup>203</sup> Smaller size MNPs are more quickly absorbed by organisms, thus accumulating more in the organism, leading to more toxic results. For example, AgNPs of 20 nm were found to be more toxic than 40 nm with a lower LC<sub>50</sub> value.<sup>179</sup>

Although smaller-sized nanoparticles have a larger surface area, the evolution of MNPs (uptake, aggregation, elimination) inside the body of an organism under study is a crucial determinant of the toxicity of variably sized nanoparticles.<sup>204</sup> Snails *B. aeruginosa* exposed to 40 nm and 80 nm AgNPs were found to show significantly enhanced oxidative stress compared to those exposed to 20 nm AgNPs.<sup>24</sup> This comprises our present meta-analysis. It can be explained by the size similarity of AgNPs around 20 nm (18.0 ± 7.7 nm) and the sediment (14.60 ± 0.19 nm), and the NPs being adsorbed readily into sediment particles, which reduces the bioavailability.<sup>24</sup> MNPs are greatly influenced by the specific NOM type or other natural particles (*e.g.*, colloids) present in freshwater. The smaller-sized NPs reacted rapidly with the substances in the solution, causing aggregation, which may have caused lower toxicity.<sup>7</sup> The mechanism behind this higher toxicity triggered by larger size remains more fully elucidated.

## 6. Development of MNP AOPs in freshwater invertebrates

Emerging numbers and diversity of chemical pollutants are urgently needed for toxicological profile access with higher

speed and accuracy, lower resource consumption and fewer experimental animals.<sup>205</sup> To address this challenge, a novel conceptual framework called the adverse outcome pathway (AOP) was proposed in 2010 by the US Environmental Protection Agency<sup>205</sup> and adopted in 2012 by OECD for ecological risk assessment.<sup>206</sup> The AOP framework focuses on identifying the biologically plausible and empirically supported links between molecular-level perturbations of a biological system caused by a stressor and an adverse outcome at a higher level of biological organization (*i.e.*, an organism or population).<sup>206</sup> Generally, the elements of the AOP include: 1) a molecular initiating event (MIE) where a stressor interacts with a biomolecule to create a perturbation; 2) a series of related key events (KEs) at the cellular, tissue, and organ levels that are caused by the MIE and are essential for the progression to an adverse outcome; and 3) adverse outcome (AO) at the organismal or population levels.<sup>7,205</sup> The MIE is known to directly trigger ROS production for nanoparticles, one of the most significant reasons for adverse MNPs effects. Likewise, oxidative stress is a known contributor to MNP-induced cell damage and toxicity.<sup>7,16,141</sup> Possible AO may be causally related to key events (KEs), for example, MNPs may lead to sequential interactions at the molecular (*e.g.*, LPO), cellular levels (*e.g.*, DNA damage, gene expression) and then the organ levels (*e.g.*, altered physiology, organ function and histopathology). These KEs may contribute to the AOs at the organism level expressed (*e.g.*, individual development, reproductive output and offspring viability) and population levels (*e.g.*, altered structure or reduced recruitment).

The AOP framework has been well developed as a tool to identify key initiators and predict effects induced by nanomaterials in mammalian species<sup>207</sup> and vertebrate species.<sup>208–211</sup> For example, PVP-AgNPs and AgNO<sub>3</sub> exposed freshwater fish *Pimephales promelas* both affected pathways involved in Na<sup>+</sup>, K<sup>+</sup>, and H<sup>+</sup> homeostasis and oxidative stress, and the MIE of Ag exposure is ROS production.<sup>210</sup> ROS production on gonad tissue of zebrafish was also identified as the MIE of AgNP exposure, and these were associated with the mitochondrion-mediated apoptosis pathway.<sup>211</sup>

Invertebrates perform essential roles in most ecosystems. Published papers have made progress in using invertebrate species as model organisms. However, MNPs' environmental impacts on invertebrates and toxicity mechanisms are yet to be fully elucidated, and the knowledge about using invertebrates in the development of AOPs remains scarce. The short life cycle offers valuable opportunities to study the impact of chemical exposure at environmentally relevant concentrations over regular periods.

To date, only a few invertebrate AOPs have been developed. Those are aimed at abundant organisms with short life cycles, such as planktonic crustaceans *Daphnia*, roundworm *Caenorhabditis elegans*, and common fruit fly *Drosophila* genus.<sup>212</sup> A recent study developed two conceptual AOPs for hazard and risk assessment of ionizing radiation and associated radionuclides to *D. magna*.<sup>213</sup> The studies





**Fig. 5** Flow diagram illustrating an AOP. The putative MIEs, KEs and AOs are illustrated based on the published research on the toxic mechanisms of nanomaterials (such as ZnONPs, CuONPs and AgNPs) in organisms. The cellular response (including oxidative stress, DNA damage and gene expression) and organ damage (*i.e.* physiology response, organic function and histopathology) are essential integrators of multiple upstream KEs. Upon the above effects, MNPs could eventually lead to sequential higher-order effects producing adverse outcomes (*i.e.* influencing the individual development, reproductive output and offspring viability). If NPs diminish the fitness of organisms, the population dynamics might be affected and possibly affect ecosystem stability and functioning.

indicated that multiple toxicity pathways were potentially involved in the  $\gamma$  radiation-mediated reproductive effects, such as the DNA damage-oocyte apoptosis pathway and the lipid peroxidation-ATP depletion pathway.<sup>213</sup>

AOPs for MNPs in environmental species are virtually absent in research papers, let alone the freshwater invertebrates. CuO NPs may generate adverse outcomes (*i.e.* reproduction impairment) to soil invertebrates *Enchytraeus crypticus* via affecting the Notch signalling pathway with consequences at cellular division and differentiation.<sup>214</sup> Considering their fundamental importance in ecosystem structure and function, investigations using key invertebrate species are vital when obtaining data from which to develop AOPs. Several aquatic invertebrates were identified as potential model organisms such as *D. magna*; rotifer, *Brachionus koreanus*; copepod *Tigriopus japonicus*, and *L. stagnalis* due to the extensive genomics information available for these species.<sup>215,216</sup>

Omics technologies, defined as high-content datasets with measurements of genes, mRNA, proteins, and metabolites, have been proved by increased studies on the potential applications of omics for ecology risk assessments.<sup>217,218</sup> It is thought-provoking how omics data can support a more predictive approach to the AOP framework, including facilitating the identification of molecular-level changes (*e.g.*, MIEs and early KEs). In real-life scenarios, omics are deployed for discovering informative biomarkers in organisms. For example, the Antczak group used transcriptomics to reveal a calcium-dependent mechanism for narcotic chemical toxicity in *D. magna*.<sup>219</sup> However, the application of omics in AOPs has not been widely investigated to date. Omics technologies, if properly applied, are likely to dramatically increase our ability to characterize from the molecular responses to the populational level. Hence, it may provide us with a unique opportunity to better recognize the consequences of MNP release into the environment. Fig. 5 shows the flow diagram illustrating an AOP, representing MNPs triggering molecular initiating events leading to a sequential series of higher order effects to produce an adverse outcome. As discussed above in

section 4, the molecular biomarkers in MNP exposure of invertebrates can help identify different MOAs on the subcellular level and thus associate them with different AOPs in environmental risk assessment.

## 7. Conclusion and perspectives

As MNP-based commodities are increasingly widespread, further studies are demanded on the effects, interactions, uptake, translocation and evolution of MNPs in organisms and the environment.<sup>220</sup> Despite significant advances in understanding the toxicity of MNPs, the field is still in its infancy, with much to be investigated.

The present review summarizes the environmental impact of MNPs and potential toxicity endpoints in freshwater invertebrates. Once released into the environment, the MNPs are transformed from their previously prepared form. This transformation remains a challenging issue for the realistic evaluation of their ecotoxicity.<sup>221</sup> Present studies show that different studies within the same subject vary enormously and even produce conflicting results. The reasons for these seemingly contradictory findings are diverse. Therefore, all possible factors should be considered as much as possible when conducting ecotoxicological studies on MNPs, *e.g.* different environmental conditions and pre-treatment methods of MNPs.

We summarise some factors that may contribute to differences in experimental results:

- (1) For the same MNP type, the particle size, crystalline shape and morphology may be different from experiments.
- (2) Different pretreatments lead to different degrees of polymerisation of MNPs in solution;
- (3) Different solvents are used.
- (4) Variation in experimental environmental conditions, such as temperature, pH, light intensity, *etc.*
- (5) Experimental organisms being different subspecies, or in different life cycles, or different routes of administration.

As the MNP research goes deeper, the following five aspects deserve additional attention:



1) Toxicity testing was performed using bivalves, gastropods and crustaceans as bioindicators and suitable model organisms and they were widely used in acute toxicity studies. However, there exist differences in the physical-chemical properties between the natural water and lab culture medium, and toxicity research in natural water needs to be considered in future investigations.

2) The chronic exposure concentrations in research materials are higher than environmental-related concentrations. Moreover, the duration of MNPs' action in the natural environment is much longer than that simulated in the laboratory. Therefore, studies on the long-term effects of low concentrations of MNPs on organisms need to be strengthened.

3) The data on the bioaccumulation of MNPs by the above-discussed model organisms are not yet perfect, as the experimental exposure concentrations vary greatly, and the methods of representing the results are not uniform, making it difficult to compare the bioaccumulation capacity between studies. Hence, there is a need to establish a standard method for MNP bioaccumulation and to optimize the establishing exposure concentration criteria; additionally, MNPs are often not entirely excreted by organisms, and the morphological transformation, long-term chronic toxicity, intergenerational transfer and trophic transport of these residual MNPs need to be studied in depth.

4) The investigations for multiple changes allow a much better understanding of toxicological pathways than the single end-point approach. Hence, there is a call to use the AOP approach to mechanistically link the molecular and cellular phenotypes to adverse organismal outcomes, determine the role of environmental stress responses at the cellular and tissue levels, and translate them into the organism's fitness consequences.

5) Genomics, transcriptomics, proteomics and metabolomics have been applied in toxicology for more than a decade and the omics approach has only been deployed in aquatic invertebrates of MNP investigations in the last few years. The integration of multi-omics data shall lead to deeper biological insights and a more comprehensive understanding of toxic mechanisms of action. In addition, multi-omics data can improve the confidence of chemical dose extrapolation, facilitate chemical identification, taxonomic characterisation and screening, and play an essential role in identifying chemical-related exposure, effect and susceptibility markers.

## Author contributions

All the authors have contributed to the discussions leading to the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

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