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REVIEW ARTICLE

# Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review

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**Abstract** Nanoparticles (NPs) of copper oxide (CuO), zinc oxide (ZnO) and especially nanosilver are intentionally used to fight the undesirable growth of bacteria, fungi and algae. Release of these NPs from consumer and household products into waste streams and further into the environment may, however, pose threat to the 'non-target' organisms, such as natural microbes and aquatic organisms. This review summarizes the recent research on (eco)toxicity of silver (Ag), CuO and ZnO NPs. Organism-wise it focuses on key test species used for the analysis of ecotoxicological hazard. For comparison, the toxic effects of studied NPs toward mammalian cells in vitro were addressed. Altogether 317 L(E)C50 or minimal inhibitory concentrations (MIC) values were obtained for algae, crustaceans, fish, bacteria, yeast, nematodes, protozoa and mammalian cell lines. As a rule, crustaceans, algae and fish proved most sensitive to the studied NPs. The median L(E)C50 values of Ag NPs, CuO NPs and ZnO NPs (mg/L) were 0.01, 2.1 and 2.3 for crustaceans; 0.36, 2.8 and 0.08 for algae; and 1.36, 100 and 3.0 for fish, respectively. Surprisingly, the NPs were less toxic to bacteria than to aquatic organisms: the median MIC values for bacteria were 7.1, 200 and 500 mg/L for Ag, CuO and ZnO NPs, respectively. In comparison, the respective median L(E)C50 values for mammalian cells were 11.3, 25 and 43 mg/L. Thus, the toxic range of all the three metal-containing NPs to target- and non-target organisms overlaps, indicating that the leaching of biocidal NPs from consumer products should be addressed.

**Keywords** Risk assessment · In vitro toxicology · Antimicrobials · Mechanism of action · REACH · QSARs

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## Introduction

Nanoindustry is one of the fastest growing industries in the history of mankind and has been referred to as the next industrial revolution (Lux Research 2008). The first national nanotechnology program—the National Nanotechnology Initiative—was launched in USA in 2000. Since then, more than 60 nations have established similar programs. In 2010, worldwide annual public and private sector funding for nanotechnologies was 17.8 billion dollars in total (Sargent 2012). As a result, the global socio-economic value of nanotechnologies is steadily increasing, and currently, nanoscale particles have significant impacts on almost all industries and all areas of society.

According to the recent review issued by the European Commission (2013), nanomaterial is defined as 'a natural, incidental or manufactured material containing particles, in

an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.' In scientific literature engineered (or manufactured or synthetic or man-made) nanoparticles (NPs) are usually defined as particles with at least one dimension between 1 and 100 nm.

At nanoscale materials have different or enhanced properties compared with their conventional 'bulk' (microsize) counterparts, due to an increased relative surface area that translates into higher reactivity (Nel et al. 2006). While in bulk materials the surface atoms constitute only a few percent of the total number of atoms, in NPs most of the atoms lay close to or at the surface (Casals et al. 2012). There is increasing evidence that the unique desired physico-chemical properties of NPs, which make nanomaterials more efficient in industrial applications, render these materials also more harmful to living organisms. Due to increasing production volumes of NPs and growing likelihood of occupational and environmental exposure to nanomaterials, the legislative bodies in both EU and USA have currently focused their activities on assessing health and environmental risks of nanotechnology.

As shown in Fig. 1, this review aims to provide a critical summary of recent scientific literature on potential hazardous effects of three types of engineered metal-containing NPs—zinc oxide (ZnO), copper oxide (CuO) and silver (Ag). All these compounds (either in the bulk or nanoform) have been historically used as biocides, that is, for avoiding or stopping the growth of microorganisms and algae (Kahru and Dubourguier 2010). Therefore, similarly to pesticides, these nanomaterials should be monitored for their toxic action also toward non-target species, including humans. In the context of the current review, 'target organism' is defined as an organism for which the biocidal NPs were designed for (e.g., bacteria and fungi as target organisms of all three NPs and algae as target organisms of CuO and Ag NPs) and 'non-target organism' is an organism which will be exposed to NPs after their incidental release into the environment. To gain a better understanding whether the accidental release of metal-containing NPs may pose a threat to non-target species, we collected toxicity data on these NPs for algae, crustaceans, fish, bacteria, yeast, nematodes, protozoa and mammalian cell lines and compared the toxicity values of NPs to target- and non-target organisms. In addition, we analyzed the collected data with respect to the correlation between the dissolution, size and coating of NPs and their toxicity to different organism groups. Finally, we classified the studied NPs into different hazard categories. However, the proposed hazard categories are rather general and could only be applied for the initial hazard identification. For complete risk assessment, further data on realistic environmental exposure scenarios for these NPs are required. Also, in case of mammalian cell lines, we do not discuss the transferability of collected in vitro data to in vivo situation.

## Production and application of Ag, CuO and ZnO (nano)particles

Estimated global production of NPs is shown in Fig. 2a (adapted from Piccinno et al. 2012). Although $SiO_2$ NPs are produced at the highest production volume (Fig. 2), Ag NPs are the ones most used in consumer products. According to the Woodrow Wilson Database (Wilson 2012), there were more than 1,300 nanotechnological consumer products on the market in March 2011, and 313 of them contained nanosilver. In consumer products, NPs are either added to the bulk material to reinforce the physical properties of the material or applied on the surface of the product to provide enhanced surface features such as scratch resistance, water repellency, reflectivity and photo activity. As the number of published articles can be considered as an early indicator of the future use of NPs, ISI Web of Science (ISI WoS) was used to gather data on the current and potential applications of Ag, ZnO and CuO NPs (Table S1 and in Fig. 2). The analysis of the collected data showed that the majority of articles concerned the applications of Ag NPs (7,699 papers, 59 %), followed by ZnO (4,640 papers, 36 %) and finally CuO NPs (690 papers, 5 %). Interestingly, the most prominent application area of all these three NPs was sensors, sensing devices and catalysis (Fig. 2b–d). Moreover, as silver is the best conductor among the metals (Ren et al. 2005) and Ag NPs have favorable chemical and physical properties such as biocompatibility, unique electronic and catalytic properties, Ag NP-based electrochemical (bio)sensing systems have been developed (Lian et al. 2013) that enable enhancing electron transfer between biomolecules (e.g., proteins) and electrode surfaces. As expected, a considerable share (19 %) of all the fields of application of Ag NPs concerned antimicrobial usage. In case of CuO NPs and ZnO NPs, this share was much lower, 4 and 2.6 %, respectively.

### Ag nanoparticles

Silver has been used to fight infections as far back as the days of ancient Greece and Egypt. In World War I, before the advent of antibiotics, silver compounds were used to prevent and treat infections. Currently, Ag NPs are the most widely commercialized NPs that are used as antimicrobials in various consumer products ranging from cosmetics,

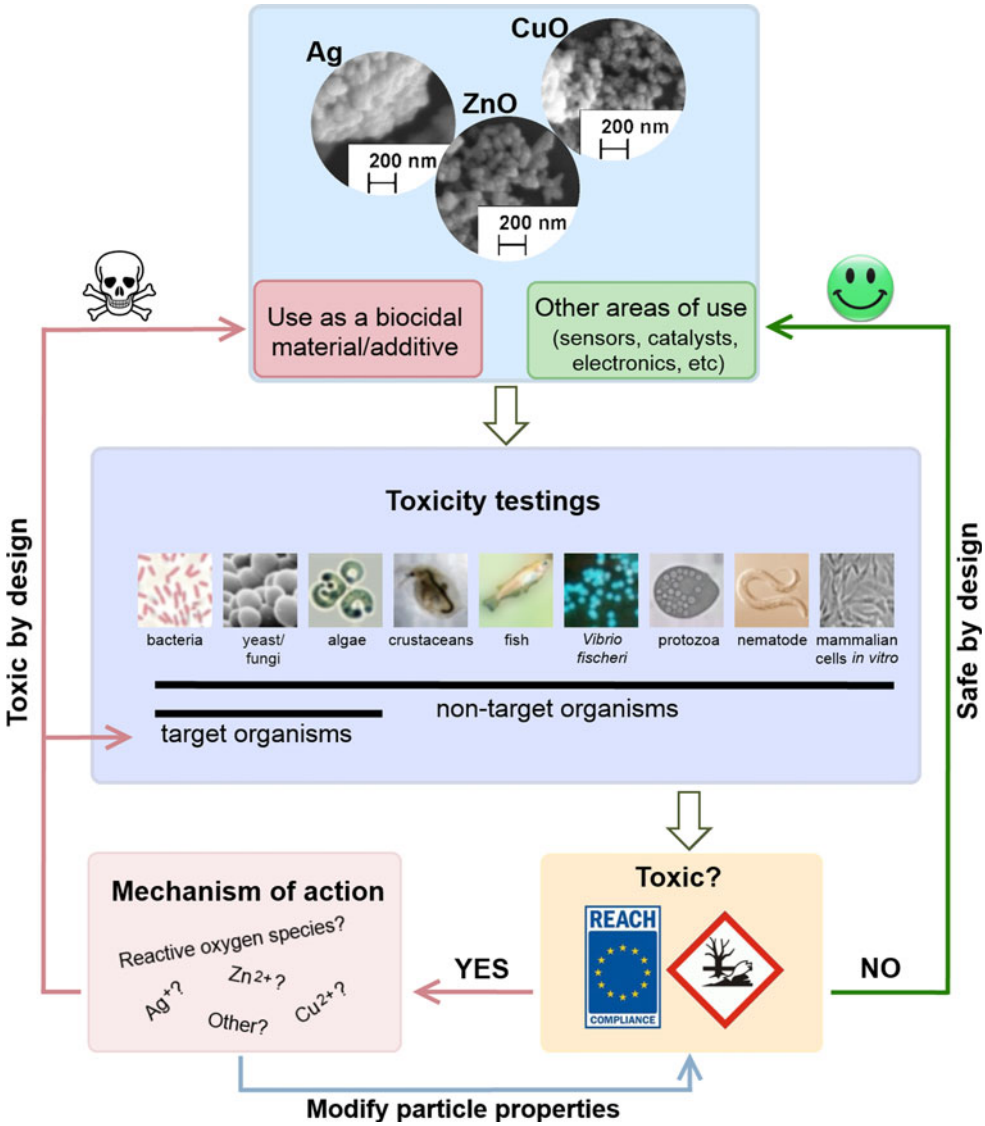


**Fig. 1** Schematic representation of the scope of the current review

clothing, shoes, detergents, dietary supplements to surface coatings in respirators, water filters, phones, laptops, toys and commercial home water purification systems such as Aquapure, Kinetico and QSI-Nano (Bystrzejewska-Piotrowska et al. 2009; Marambio-Jones and Hoek 2010; Cerkez et al. 2012). In addition to antibacterial, antiviral and antifungal properties (for the review and references therein, see Ivask et al. 2012), nanosilver has also been shown to facilitate wound healing (Nair and Laurencin 2007). Estimated global annual production of Ag NPs is ~55 tons (a median value; Piccinno et al. 2012; Fig. 2a).

## ZnO nanoparticles

According to different sources, the worldwide annual production of ZnO NPs is estimated to be between 550 (Piccinno et al. 2012; Fig. 2d) and 33,400 tons (Research and Markets 2012). Thus, among metal-containing NPs, ZnO NPs have the third highest global production volume after $SiO_2$ and $TiO_2$ NPs (5,500 and 3,000 tons annually, respectively) (Piccinno et al. 2012; Fig. 2a). ZnO NPs are mostly used as a UV light scattering additive in cosmetics such as sunscreens, toothpastes and beauty products (Serpone et al. 2007). ZnO NPs are widely used in rubber manufacture, production of solar cells and LCDs, pigments (as a whitener), chemical fibers, electronics and textiles (Dastjerdi and Montazer 2010; Song et al. 2010). In addition, ZnO is an essential ingredient in almost all types of antifouling paints (IPPIC 2012), and recently bulk ZnO has been increasingly replaced by ZnO NPs because of their enhanced antibacterial properties (Padmavathy and Vijayaraghavan 2008).

## CuO nanoparticles

In contrast to Ag and ZnO NPs, we were not able to retrieve data on the current production volumes of CuO

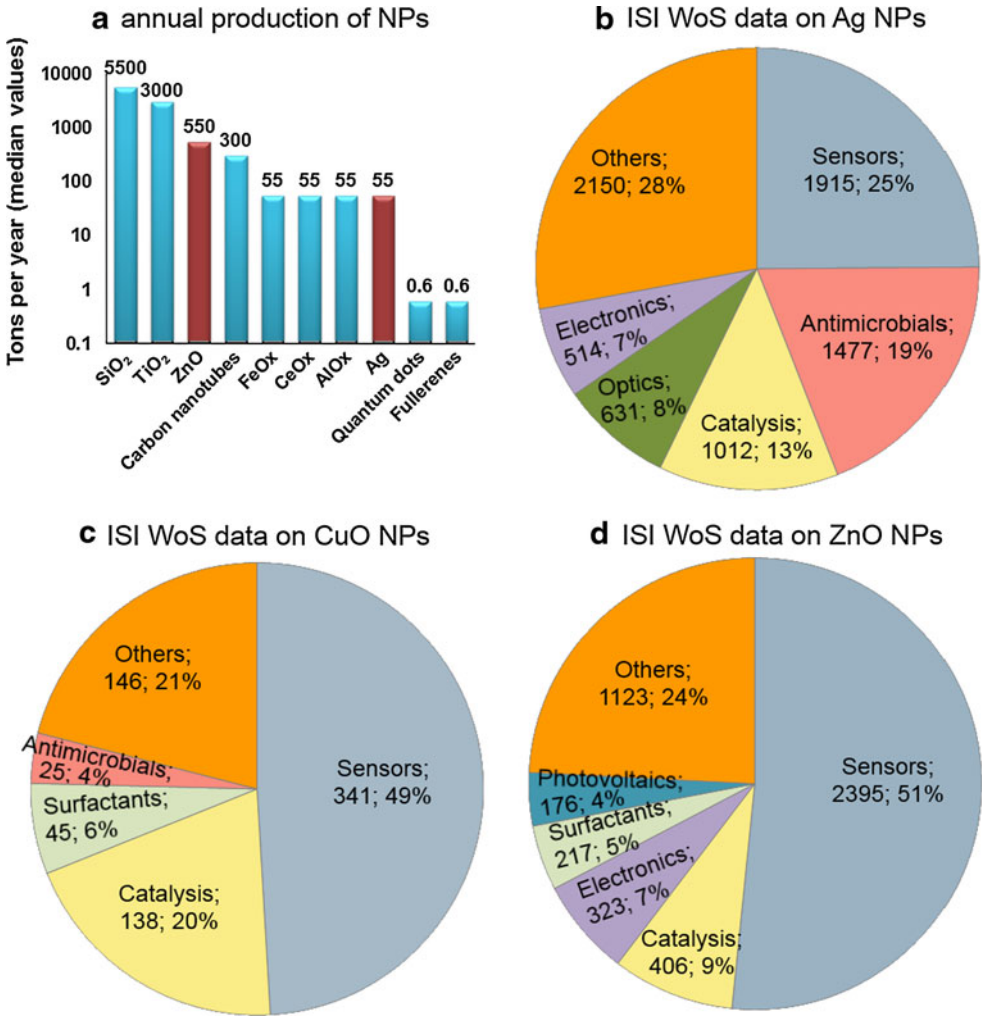


**Fig. 2** **a** Annual production volumes of nanomaterials (data are adapted from Piccinno et al. 2012). **b–d** Fields of application of Ag (**b**), CuO (**c**) and ZnO (**d**) nanoparticles based on the publications indexed by Thomson Reuters ISI Web of Science. Search was done in March 2013. The following search terms were used: 'silver' OR 'CuO' OR 'ZnO' AND 'nano*' AND 'application category' (indicated in the figure). Numbers next to each application category indicate the number of articles retrieved and their respective percent share. The numerical data are presented in Supplementary Table S1

NPs. As these NPs are used in lower quantities and compared to other NPs the potential hazardous effects of CuO NPs are poorly studied (Kahru and Savolainen 2010), it is reasonable to conclude that they are also manufactured in lower amounts compared to other NPs. As reflected by Fig. 2c, the most important and unique application area of CuO NPs is electronics and technology (semiconductors, electronic chips, heat transfer nanofluids), as CuO has excellent thermophysical properties (Ebrahimnia-Bajestan et al. 2011). Also other applications such as gas sensors (Li et al. 2007), catalytic processes (Carnes and Klabunde 2003), solar cells and lithium batteries (Guo et al. 2009; Sau et al. 2010) have been suggested for CuO NPs. CuO NPs have been shown to inhibit the growth of microorganisms and exert antiviral properties (Borkow and Gabbay 2004; Gabbay et al. 2006). For these reasons, CuO NPs have been used in face masks, wound dressings and socks to give them biocidal properties (Borkow et al. 2009, 2010a, b).

## The need for toxicity data on ZnO, CuO and Ag (nano)particles

### Toxicity data and data quality gaps for nanoparticles

The scientific information on potential harmful effects of NPs severely lags behind the development of nanotechnologies (Shvedova et al. 2010; Kahru and Ivask 2013). In addition, the available nanotoxicity data are inconsistent because experimental approaches vary from article to article making it impossible to compare results (Schrurs and Lison 2012). To overcome these problems, nanotoxicology community has recently started a discussion about the implementation of general guidelines for nanotoxicology research and establishment of common parameters that should be addressed in all nanotoxicological articles (Nature Nanotech Editorial 2012).

Legislation gaps for nanoparticles

Currently, the production and use of nanoparticle-containing products is not internationally regulated by any distinct safety regulation (EC 2008). Compared to bulk materials, NPs have unique physico-chemical properties such as higher stability in the aquatic environment (Fig. 3b), decreased size (Fig. 3c) and increased specific surface area (SSA), and thus enhanced reactivity. These properties make NPs more efficient and interesting for different industrial applications but at the same time make them more harmful to living organisms. Thus, theoretically a special guidance should be considered for NPs. Yet, as NPs are chemically identical to their bulk counterparts and thus have the same CAS number (Fig. 3a), they are not recognized by industry as a new class of chemicals. As a result, the production and use of metal-containing NPs are subject to analogous regulation as the conventional bulk chemical compounds regulated in Europe by EU chemical safety policy REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). The REACH regulation states that when chemicals/NPs are produced in a volume of more than one ton per year and sold at the European market, they must be characterized for their potential impact on aquatic ecosystems (European Parliament 2006). The data provided by the producer/importer should include short-term (48 h) toxicity testing on crustaceans (preferred species *Daphnia magna*, OECD 2004) and 72h growth inhibition of aquatic plants (preferably algae, OECD 2011). In addition, short-term (96 h) toxicity testing on fish (OECD 1992) is required at the next annual tonnage level (>10 tons per year). As shown in Crane et al. (2008), Kahru et al. (2008) and Kahru and Dubourguier (2010), the types of test species and biological endpoints used within standard environmental hazard assessment frameworks are generally appropriate also for nanoecotoxicological purposes. The additional specific requirements for NP studies are the dispersion conditions and characterization of the particles in the test environment as well as careful consideration of test conditions for potential artifacts that can arise due to the color of NPs or their sorptive properties (Handy et al. 2012; Schrurs and Lison 2012; Bayat et al. 2013). Analogously to the rest of the chemical compounds, NPs are classified with respect to their environmental toxicity according to the response of the most sensitive of the three test organisms: algae, crustaceans and fish (European Union 2011).

## Specific physico-chemical properties of metal-containing nanoparticles

In order to understand the mechanisms behind the toxicity of NPs, the physico-chemical properties of the particles should be thoroughly analyzed in relevant test environments. Recent review by Bandyopadhyay et al. (2012) gives an in-depth overview of the methods that can be applied to characterize NPs size, shape, crystal structure, aggregation, chemical composition, surface properties (surface charge, area, chemistry), solubility and porosity. Since detailed reviews about characterization of the NPs can be found elsewhere, the following paragraphs of this review focus on joint nominators and differences in the physico-chemical characteristics of Ag, CuO and ZnO NPs.

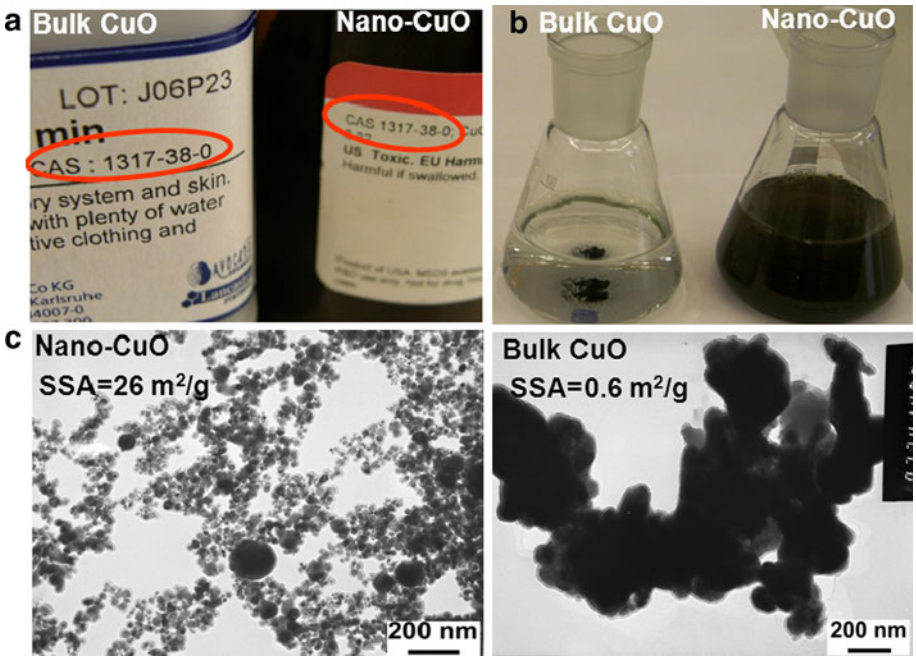


**Fig. 3** **a** Labels of bulk CuO and nanosized CuO. Note the same CAS number. **b** 200 mg/L stock suspensions of CuO. **c** TEM image of nano CuO and bulk CuO. Note 43-fold difference in the SSAs of bulk CuO and nanosized CuO

### Joint nominators for Ag, CuO and ZnO nanoparticles

Considering the joint nominators for Ag, CuO and ZnO NPs, the first to notice is the metallic elemental composition of all the three selected particles. Secondly, all the three NPs are applied to fight the undesirable growth of microorganisms. Although among the three nanomaterials, silver NPs are used most widely as antimicrobials, also CuO and ZnO NPs have been successfully used as biocides (Fig. 2c–d). The third joint nominator for the three NPs is their negative surface charge, which results from oxygen atoms in CuO and ZnO (Xu et al. 2012). Though Ag NPs do not initially contain oxygen, the surface of metallic Ag NPs is oxidized under most environmental conditions (aerobic) and negatively charged hydroxo and oxo groups cause the negative surface charge of the particle (Levard et al. 2012). The fourth and toxicologically perhaps the most important joint property is that all the three NPs are soluble to some extent in aqueous media. We have previously shown that the solubility of CuO and ZnO NPs is the key issue in the toxicity of metal-containing (nano)particles and stressed that the solubility data reported as N/A (not available or not applicable) in Material Safety Data Sheet (MSDS) of ZnO and CuO NPs should be addressed (Aruoja et al. 2009; Ivask et al. 2010; Bondarenko et al. 2012). It has been also emphasized that aqueous solubility of NPs has to be incorporated into the environmental risk assessment models of NPs in addition to other key physico-chemical characteristics relevant to NPs (European Commission 2007). Solubility of NPs and the behavior of released metal ions, that is, the proportion of intact particles, metal ions and metal complexes, depend greatly on the properties of the test environment (for a review and references therein, see Casals et al. 2012). The most important parameters of the test environment are pH, dissolved organic carbon content and water hardness (Wiench et al. 2009; Fabrega et al. 2011). For instance, the solubility of all the three selected particles is enhanced at more acidic pH (Dimkpa, et al. 2011; Fabrega et al. 2011; Levard et al. 2012; Ma et al. 2013). Also, the solubility of the aforementioned NPs depends on their interactions with organic material in the test environment (proteins, amino acids, natural organic matter, humic substances) that may coat and disperse NPs or complex metal ions. For example, reduced solubility and toxicity toward crustaceans has been observed in natural waters for Ag NPs (Gao et al. 2009) and CuO NPs (Blinova et al. 2010).

Figure 4 illustrates the behavior of NPs in various test environments: in all test media *coated* Ag NPs are remarkably more stable than the *uncoated* NPs. That is coherent with the results by Fabrega et al. (2011) showing that in high ionic strength suspensions uncoated Ag NPs tend to precipitate and sediment within a few hours after the start of the toxicity assay. Also, CuO and ZnO NPs were remarkably unstable and tended to sediment. Figure 4 also shows that the agglomeration/sedimentation of CuO and ZnO was especially high in mineral media—media that are used for key regulatory ecotoxicological assays (crustaceans, algae) described above. In contrast, the components of the complex test media (defined here as the test environment with organic components) dispersed NPs and prevented their sedimentation. In addition, the complex media may promote dissolution of NPs (Käkinen et al. 2011; Kasemets et al. 2013).

In summary, as also underlined in the recent paper by Casals et al. (2012), it is extremely important to assess the physico-chemical properties of NPs in the media where the biological toxicity tests are performed. As dissolution is one of the main contributors to the toxicity of Ag, CuO and ZnO NPs, in this review their toxicity is discussed in parallel with the toxic effects of the respective ions.

### Differences between Ag, CuO and ZnO nanoparticles

In addition to the above-described *joint nominators*, there are also *differences* between the three NPs selected for this study. To begin with, their chemical composition is different; thus, in similar particle size their toxicity is likely different (Sharifi et al. 2012). In addition, copper is a redox element having common valences of +2 or +1. Thus, differently from zinc and silver, redox-active Cu ions may also be involved in electron-transfer processes. Third, the surface of Ag NPs but not CuO and ZnO NPs is frequently functionalized with different coatings, polyvinylpyrrolidone (PVP) and citrate being the most widely used. Last but not least, copper and zinc (but not silver) are necessary trace elements for almost all types of living cells, while silver has no known function in the living organisms (Sandstead 1995).

## Toxicity of Ag, CuO and ZnO nanoparticles to target and non-target organisms

The review by Crane et al. (2008) summarizes various OECD assays that can be applied for the toxicity testing of NPs. Assessment of the environmental hazard of NPs under REACH regulation requires that at least two OECD tests with algae (OECD201) and crustacean *D. magna* (OECD202) should be used. In this review, we collected, analyzed and summarized the toxicity data (including but not limited to the key OECD test species) from the published literature on ZnO, CuO and Ag NPs.

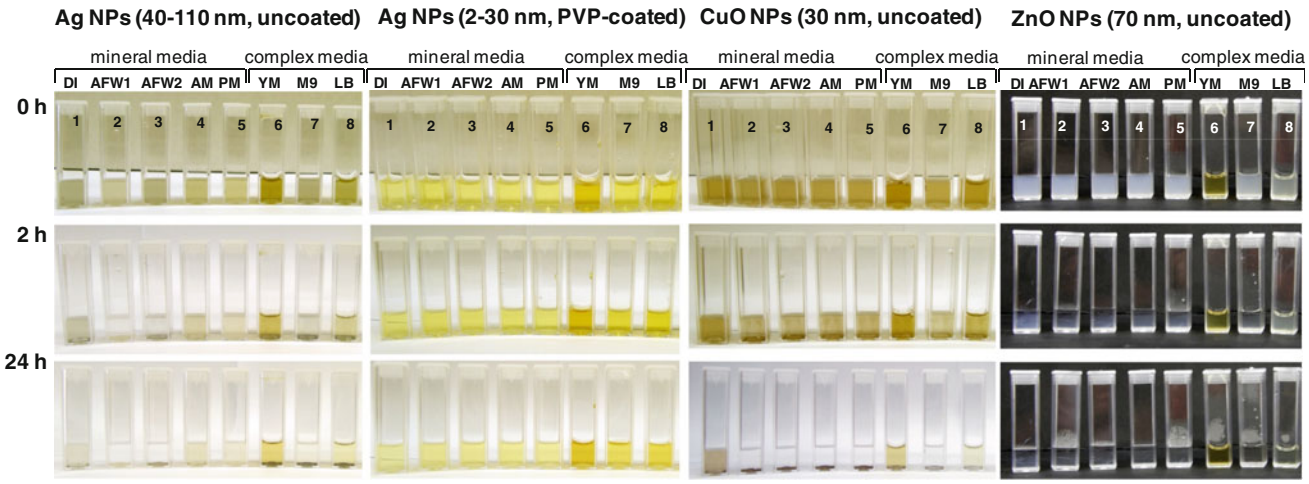


**Fig. 4** Uncoated Ag (50 mg/L), PVP-coated Ag (50 mg/L), uncoated CuO (50 mg/L) and ZnO NPs (200 mg/L) after 0, 2 and 24 h incubation in different (eco)toxicological test environments: *1* deionized water; *2* artificial freshwater for the tests with *Daphnia* sp. (OECD 202); *3* AFW for *Thamnocephalus* sp. (Thamnotoxkit F$^{TM}$ 1995); *4* algal growth medium (OECD 201); *5* protozoan mineral test medium (Osterhout's); *6* yeast extract peptone dextrose medium; *7* bacterial M9 medium supplemented with 0.1 % glucose and 0.5 % amino acids; *8* bacterial LB medium containing tryptone and yeast extract. Detailed composition of test media is given in Käkinen et al. (2011)

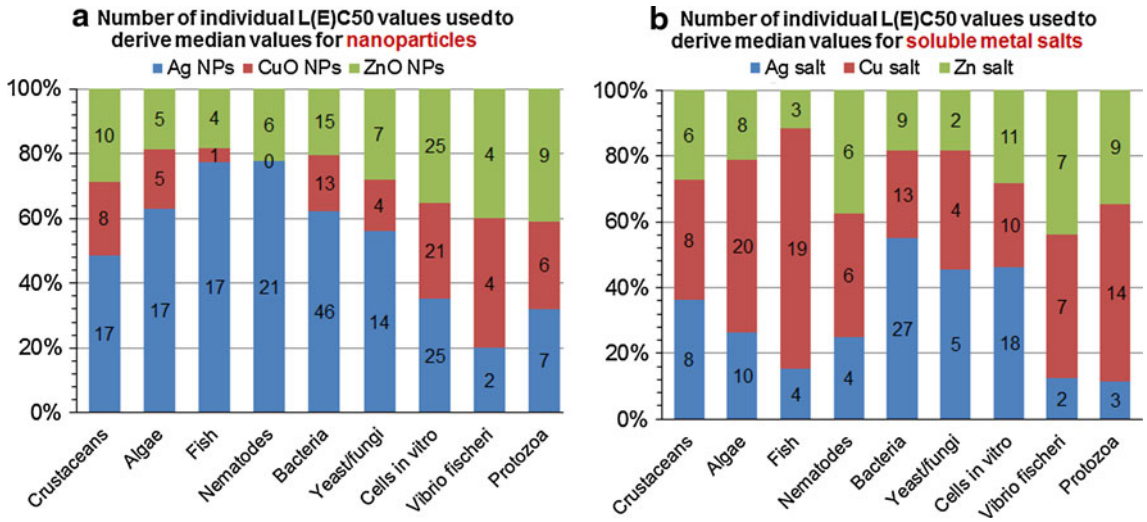


**Fig. 5** Number and share of individual L(E)C50 or MIC values used to derive the median L(E)C50 or MIC for nanoparticles (**a**) and metal salts (**b**). Total number of individual values: 317

## Characterization of retrieved toxicity data set

When collecting the toxicity data for Ag, CuO and ZnO NPs, we relied on recent nano(eco)toxicological peer-reviewed literature that preferably contained data not only on toxicity of NPs but also physico-chemical characteristics of the studied NPs prior to and during toxicity testing. Our goal was to find at least 10 quantitative toxicity values (EC50, LC50, MIC) per organism and NP type. In parallel, we collected toxicity data for metal ions to assess the impact of dissolution on toxicity of NPs. Organism-wise we focused on bacteria, crustaceans, algae, fish, nematodes, yeasts, protozoa as well as on mammalian cell lines.

Figure 5 shows the availability of the toxicity data in ISI WoS. As can be seen, relatively large amount of data was available on toxicity of Ag NPs, whereas less information was published on toxicity of ZnO NPs and the data on CuO were especially scarce. At the same time, there was a lot of data on the toxicity of both Cu and Ag ions, while less information was available on the toxicity of Zn ions.

Table S2 presents data on the test organisms that were used most often for determining the L(E)C50 and MIC values in the analyzed literature. As shown in Table S2, the

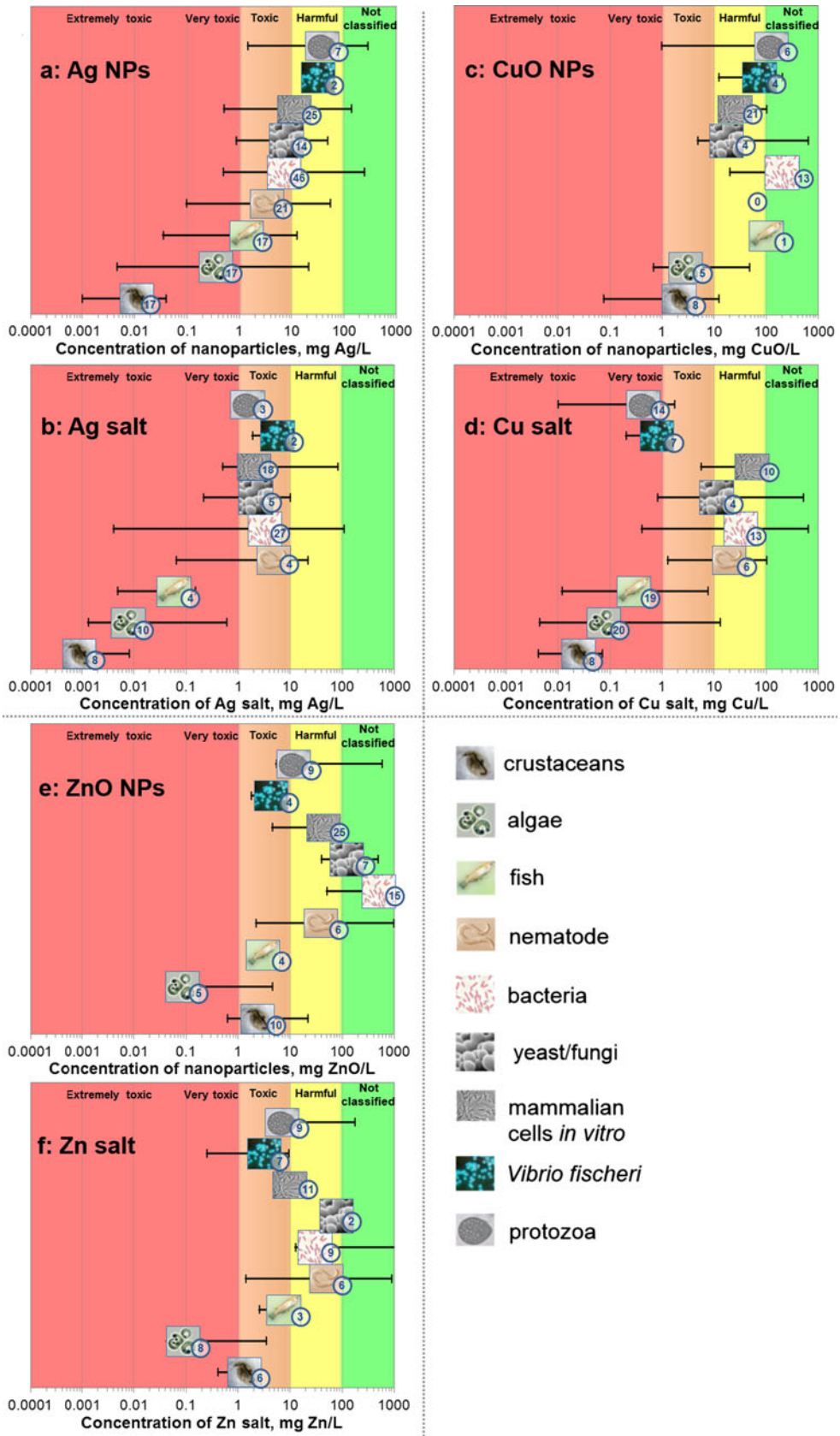


**Fig. 6** Toxicity of CuO, ZnO and Ag nanoparticles to different organisms. Median L(E)C50 values for all other organisms except bacteria and MIC for bacteria ± minimum and maximum values are presented. Different organisms/cells are shown by respective pictograms and the number on the pictogram indicates the number of L(E)C50 values used to derive the median value. Note the logarithmic scale of *x*-axis and that L(E)C50 and MIC values of NPs reflect nominal concentrations. The classification to hazard categories is explained in Table 1

**Table 1** Median L(E)C50 values for all organisms except bacteria and median MIC for bacteria for Ag, CuO and ZnO nanoparticles (NPs) and the respective metal salts

| Group of organisms | Median L(E)C50 or MIC, on compound basis, mg/L (number of data)* | | | Median L(E)C50 or MIC, on metal basis, mg metal/L (number of data)* | | |
|---|---|---|---|---|---|---|
| | Ag NPs | CuO NPs | ZnO NPs | Ag salt | Cu salt | Zn salt |
| Crustaceans | 0.01 (17) | 2.1 (8) | 2.3 (10) | 0.00085 (8) | 0.024 (8) | 1.3 (6) |
| Algae | 0.36 (17) | 2.8 (5) | 0.08 (5) | 0.0076 (10) | 0.07 (20) | 0.09 (8) |
| Fish | 1.36 (17) | 100 (1) | 3.0 (4) | 0.058 (4) | 0.28 (19) | 7.5 (3) |
| Nematodes | 3.34 (21) | Not found (0) | 39 (6) | 4.8 (4) | 19.4 (6) | 49 (6) |
| Bacteria | 7.10 (46) | 200 (13) | 500 (15) | 3.3 (27) | 32 (13) | 30 (9) |
| Yeast | 7.90 (14) | 17 (4) | 121 (7) | 2.16 (5) | 11.1 (4) | 78 (2) |
| Mammalian cells in vitro | 11.3 (25) | 25 (21) | 43 (25) | 2 (18) | 53 (10) | 9.8 (11) |
| *V. fischeri*[a] | 32 (2) | 73.6 (4) | 4.3 (4) | 5.7 (2) | 0.78 (7) | 3.2 (7) |
| Protozoa | 38 (7) | 124 (6) | 11.7 (9) | 1.5 (3) | 0.43 (14) | 7 (9) |
| Lowest L(E)C50, MIC | 0.01 | 2.1 | 0.08 | 0.00085 | 0.024 | 0.09 |
| Most sensitive organisms | Crustaceans | Crustaceans | Algae | Crustaceans | Crustaceans | Algae |
| Classification (EU-Directive 93/67/EEC (CEC 1996)[b] | Very toxic | Toxic | Very toxic | Very toxic | Very toxic | Very toxic |
| Classification (Sanderson et al. 2003; Blaise et al. 2008)[c] | Extremely toxic | Toxic | Extremely toxic | Extremely toxic | Extremely toxic | Extremely toxic |

* In the brackets next to the median value, the number of data used to derive the median value is presented

Data are summarized from Supplementary Tables S3–S8 and are arranged throughout according to the decreasing sensitivity (increasing median L(E)C50 values) of test organisms to silver nanoparticles. The L(E)C50 and MIC numbers are from the following articles: Borovanský and Riley (1989), Ershov et al. (1997), McCloskey et al. (1996), Lin et al. (1996), Zhao et al. (1998), Mobley et al. (1999), Mastin and Rodgers (2000), Grass and Rensing (2001), Franklin et al. (2002), Graff et al. (2003), Harmon et al. (2003), Teitzel and Parsek (2003), Yilmaz (2003), De Boeck et al. (2004), Hsieh et al. (2004), Jonker et al. (2004), de Oliveira-Filho et al. (2004), Shakibaie and Harati (2004), Apte et al. (2005), Cho et al. (2005), Heijerick et al. (2005), Lee et al. (2005), Chen et al. (2006), Hiriart-Baer et al. (2006), Jeng and Swanson (2006), Kungolos et al. (2006), Madoni and Romeo (2006), Panáček et al. (2006), Dechsakulthorn et al. (2007), Franklin et al. (2007), Gallego et al. (2007), Zhang et al. (2007), Calafato et al. (2008), Griffitt et al. (2008), Heinlaan et al. (2008), Hernández-Sierra et al. (2008), Jin et al. (2008), Karlsson et al. (2008), Kim et al. (2008), Martínez-Castanón et al. (2008), Mortimer et al. (2008), Navarro et al. (2008), Padmavathy and Vijayaraghavan (2008), Ruparelia et al. (2008), Zhu et al. (2008), Aruoja et al. (2009), Chae et al. (2009), Foldbjerg et al. (2009), Jain et al. (2009), Kasemets et al. (2009), Kim et al. 2009a, b, Kvitek et al. (2009), Lewis and Keller (2009), Lin et al. (2009), Liu et al. (2009), Ma et al. (2009), Oliva et al. (2009), Park and Heo (2009), Pavlica et al. (2009), Sovova et al. (2009), Teodorovic et al. (2009), Wang et al. (2009), Zhu et al. (2009), Ahamed et al. (2010), Baker et al. (2010), Blinova et al. (2010), Chen et al. (2010), Contreras et al. (2010), Ebrahimpour et al. (2010), Kennedy et al. (2010), Kim et al. (2010), Laban et al. (2010), Liu et al. (2010), Meyer et al. (2010), Miao et al. (2010), Mortimer et al. (2010), Nowrouzi et al. (2010), Panjehpour et al. (2010), Song et al. (2010), Suresh et al. (2010), Wang and Guan (2010), Wong et al. (2010), Alsop and Wood (2011), Bao et al. (2011), Dua et al. (2011), Emami-Karvani and Chehrazi (2011), Foldbjerg et al. (2011), He et al. (2011), Kim et al. (2011), Kurvet et al. (2011), Lipovsky et al. (2011), Ma et al. (2011), Majzlik et al. (2011), McLaughlin and Bonzongo (2011), Mortimer et al. (2011), Murphy et al. (2011), Naddafi et al. (2011), Niazi et al. (2011), Poynton et al. (2011), Xie et al. (2011), Xiong et al. (2011), Yu et al. (2011), Zhao et al. (2011), Albers et al. (2012), Ansari et al. (2012), Binaeian et al. (2012), Blinova et al. (2012), Brandt et al. (2012), Böhmert et al. (2012), Cao et al. (2012), Ellegaard-Jensen et al. (2012), Govindasamy and Rahuman (2012), Greulich et al. (2012), Haase et al. (2012), Harrington et al. (2012), Hassan et al. (2012), He et al. (2012), Hoheisel et al. (2012), Jo et al. (2012), Kashiwada et al. (2012), Kennedy et al. (2012), Kim et al. (2012), Kwok et al. (2012), Li et al. (2012a, b) Lim et al. (2012), Little et al. (2012), Manusadžianas et al. (2012), Monteiro et al. (2012), Oukarroum et al. (2012), Patra et al. (2012), Perreault et al. (2012), Piret et al. 2012a, b, Poynton et al. (2012), Rallo et al. (2012), Seiffert et al. (2012), Shaw et al. (2012), Shi et al. (2012), Unger and Lück (2012), Vargas-Reus et al. (2012), Wang et al. (2012a,b), Wu et al. (2012), Yang et al. (2012), Zhang et al. (2012a, b), Zhao et al. (2012), Zhao and Wang (2012), Debabrata and Giasuddin (2013), Juganson et al. (2013), Kasemets et al. (2013), Wu and Zhou (2013)

[a] *V. fischeri* data were retrieved separately from other bacteria, because *V. fischeri* (also an ISO (2010) test organism) was considered as non-target aquatic species

[b] Classification of NPs and their soluble salts to hazard categories adheres to EU-Directive 93/67/EEC (CEC 1996) and is based on the lowest median L(E)C50 value of the three key environmental organisms: algae, crustaceans and fish. <1 mg/L = very toxic to aquatic organisms; 1–10 mg/L = toxic to aquatic organisms; 10–100 mg/L = harmful to aquatic organisms; >100 mg/L = not classified

[c] Analogous to classification of CEC (1996) except that one category is added: <0.1 mg/L = extremely toxic to aquatic organisms

main representative species among crustaceans was *D. magna*, among algae *Pseudokirchneriella subcapitata,* among nematodes *Caenorhabditis elegans*, among bacteria *Escherichia coli* and among yeasts *Saccharomyces cerevisiae*. In all other groups, the dominant organism/cell type varied depending on NP type.

Altogether 317 L(E)C50 or minimal inhibitory concentrations (MIC) values for studied NPs were retrieved. Most of the data on crustaceans, algae and fish were obtained using standardized test methods. However, the protocols of bacterial, yeast, nematode and mammalian cell assays varied considerably. Most of the retrieved data represented $EC/LC_{50}$ values except for bacteria where MIC values were collected as more relevant for indicating the antimicrobial properties of NPs.

## Analysis of retrieved toxicity data set

Figure 6 depicts the median L(E)C50 or MIC values and the respective variation scale for the selected NPs and the respective soluble metal salts toward different groups of organisms/cells. Table 1 provides numerical median L(E)C50 values and the number of individual values used to derive the median value. The individual L(E)C50 values are shown in Supplementary Tables S3–S8.

Classification of NPs and soluble metal salts to different hazard categories was performed according to EU-Directive 93/67/EEC. This classification scheme is based on the lowest median L(E)C50 value of the three key environmental organisms: algae, crustaceans and fish (CEC 1996). The lowest median L(E)C50 value <1 mg/L classifies chemical as very toxic to aquatic organisms; 1–10 mg/L = toxic to aquatic organisms; 10–100 mg/L = harmful to aquatic organisms; >100 mg/L = not classified (CEC 1996). An additional category 'extremely toxic' applied by Sanderson et al. (2003) and Blaise et al. (2008) was also employed in the current review. Note that according to EU-Directive 93/67/EEC, the lowest EC50 value obtained either in tests with crustaceans, algae or fish will determine the final hazard class of the chemical compound (Table 1).

Ag NPs exhibited the highest toxicity to the crustaceans with median L(E)C50 value of 0.01 mg/L, that is, according to the most sensitive organism of the test battery crustaceans–algae–fish, Ag NPs should be classified as 'very toxic' to aquatic organisms (CEC 1996). The toxicity of Ag NPs to algae was slightly lower (median L(E)C50 = 0.36 mg/L), followed by fish, nematodes, bacteria, yeast, various mammalian cells, *Vibrio fischeri* and protozoa (Fig. 6a; Table 1). Thus, Ag NPs that are mostly used in antimicrobials and in algaecides (Nowack et al. 2011) were the most toxic toward non-target aqueous organisms—the crustaceans that are crucial components of the aquatic food web. Toxicity data of Ag NPs on bacteria, aquatic organisms and eukaryotic cells in vitro was also recently summarized by Chernousova and Epple (2013). Similarly to our findings (Table 1), these authors showed that the MIC values of Ag NPs to bacteria were in the range of 0.1–20 mg/L and to eukaryotic cells in vitro in the range of 10–100 mg/L.

It is noteworthy that the sensitivity pattern of different organisms to studied metal-containing NPs largely followed the pattern of their sensitivity to the respective metal ions. For instance, similarly to the tendency noted with Ag NPs, crustaceans, algae and fish proved the most sensitive organisms also to Ag ions (Fig. 6b; Table 1). As a rule, the difference between the L(E)C50 values of Ag NPs and Ag ions was 10–15 times (Fig. 7a), with the exception of nematode *C. elegans* for which the toxicity of Ag NPs and Ag ions, was nearly the same. However, most of the toxicity data on Ag NPs to *C. elegans* originate from the study of Yang et al. (2012), who utilized a set of toxic Ag NPs that were prepared in-house. Thus, it is difficult to conclude whether increased toxicity of Ag NPs compared to Ag ions was determined by the specific properties of Ag NPs prepared by Yang et al. (2012) or whether Ag NPs in general have more prominent particle-specific effects in *C. elegans*.

Similarly to Ag NPs, also CuO NPs were the most toxic to crustaceans and algae, but at a slightly higher level: median L(E)C50 values were around 2–3 mg CuO/L

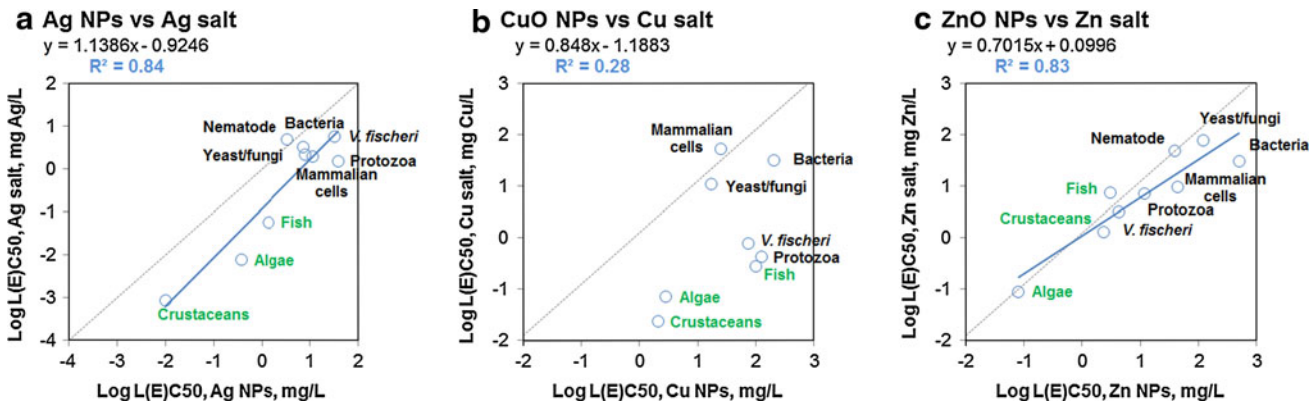


**Fig. 7** Plots of the median L(E)C50 values of Ag, CuO and ZnO NPs versus the median L(E)C50 values of the respective soluble metal salts to different organism groups. Data are plotted from Table 1

(Fig. 6c; Table 1). Thus, according to the most sensitive organism of the test battery crustaceans–algae–fish, CuO NPs should be classified as 'toxic' to aquatic organisms (CEC 1996). As a rule, in all other ecotoxicological organisms, CuO NPs exerted toxicity at relatively high nominal concentrations (L(E)C50 > 100 mg/L). As CuO NPs are also used as antibacterials (Fig. 2c), it is interesting to note that bacteria proved not sensitive toward CuO NPs (MIC > 250 mg/L). On one hand, the insensitivity of bacteria toward CuO NPs may be explained by the differences in the test media and toxicity endpoints used. Indeed, in the toxicity assays with crustaceans and algae a mineral medium with low potential for complexing of Cu ions was utilized, whereas the bacterial inhibition assays (for MIC calculation) were mostly performed in organic media with high potential for complexing of Cu ions. On the other hand, the bacterial MIC values were very similar to EC50 values collected for bioluminescent aquatic bacterium *V. fischeri* where the assay was performed in 2 % NaCl (ISO 2010). Thus, apparently CuO NPs are indeed substantially more toxic to crustaceans and algae than to bacteria, and their use as antimicrobials should be perhaps re-considered due to the ecotoxicological concerns during the 'life cycle' of CuO NP-containing products.

Cu ions were more toxic than CuO NPs to all organisms except for yeast and mammalian cells in vitro (Figs. 6d, 7b). This is an important finding showing that in mammalian cells in vitro, CuO NPs may have an additional particle-specific intrinsic toxicity that is hard to predict using non-mammalian cell models. One may hypothesize that the particles are endocytosed (a Trojan horse model) and when already inside the cell their solubilization cannot be controlled by the mechanisms used to regulate the concentration of Cu ions in the cell. On the other hand, the toxicity assays with mammalian cells in vitro use serum that may disperse and coat NPs (Zook et al. 2012) increasing their bioavailability to the cells. For yeast *S. cerevisiae*, it was shown that while the toxicity tests were done in protein-rich medium, CuO NPs enhanced the Cu-ion-associated stress assumingly due to the stronger sorption of protein-coated NPs onto the cell surface that was suggested to facilitate the dissolution of CuO in the close vicinity of the yeast cell wall. Interestingly, this effect was prominent in complex organic medium, but not in distilled water (Kasemets et al. 2013).

As in case of Ag and CuO NPs, the toxicity of ZnO NPs to algae (median L(E)C50 = 0.08 mg/L) crustaceans (L(E)C50 = 2.3 mg/L) and fish (median L(E)C50 = 3.0 mg/L) was remarkably higher than to bacteria (MIC 622 mg/L). Thus, according to the most sensitive organism of the test battery crustaceans–algae–fish, ZnO NPs should be classified as 'very toxic' to aquatic organisms (CEC 1996).

The toxicity of ZnO NPs and Zn ions to different organisms was stunningly similar (Figs. 6e–f, 7c; Table 1), indicating that the toxicity of ZnO NPs is largely caused by dissolved Zn. To further illustrate the role of dissolution in the toxicity of studied NPs, the toxicity of NPs to various organisms was plotted against the toxicity of the respective metal ions. As shown in Fig. 7, the L(E)C50 values of Ag and ZnO NPs correlated well with the respective values of the soluble salts ($R^2 = 0.84$ and 0.85, respectively). However, the plot of the L(E)C50 values of CuO NPs and Cu ions formed two clusters, distinguishing mammalian cells, yeast and bacterial cells from all other organisms. As discussed above, this was most probably caused by the test

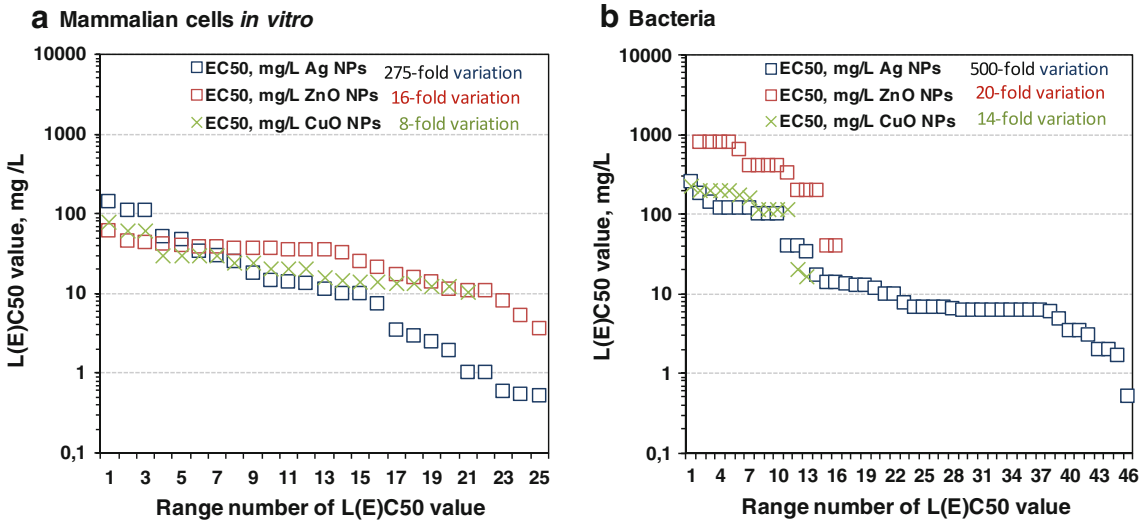


**Fig. 8** Variation in individual L(E)C50 or MIC values used to derive the median L(E)C50 or MIC value for mammalian cells in vitro (**a**) and bacteria (**b**)

environment rich in organic compounds, where organic matter enhanced dispersion of CuO NPs and increased their bioavailability to the cells.

## Variability of the retrieved toxicity data

Finally, we analyzed the obtained toxicity data with respect to the size and coating of NPs. As most of the literature data were available for bacterial cells (74 MIC values were retrieved, Fig. 6) and mammalian cells in vitro (71 EC50 values were retrieved, Fig. 6), the comparative analysis of particle size, coating and toxicity to these two cell types was performed. In addition, the toxicity mechanisms of NPs to these cell types are supposedly different, because mammalian cells internalize NPs and bacteria are more 'resistant' to the intracellularization of NPs, although some researchers have reported the penetration of NPs also into bacterial cells (Morones et al. 2005; McQuillan et al. 2012). The toxicity data of NPs to both mammalian and bacterial cells were supposed to vary because of the heterogeneity of bacterial strains and cell lines used (Table S2).

Surprisingly, we observed that the toxicity data of CuO and ZnO NPs to both groups, mammalian and bacterial cells, varied in quite narrow range: 16-fold and 20-fold for ZnO NPs and 8-fold and 14-fold for CuO NPs, respectively (Fig. 8).

**Table 2** Characterization of sizes of NPs of Ag, CuO and ZnO used to derive the median MIC values in bacterial studies or L(E)C50 values in mammalian cell in vitro studies

| | Mammalian cells in vitro | | | Bacteria | | |
|---|---|---|---|---|---|---|
| | Ag | CuO | ZnO | Ag | CuO | ZnO |
| Nr of data | 28 | 22 | 25 | 46 | 13 | 15 |
| Maximum size, nm | 69 | 55 | 1000 | 89 | 30 | 125 |
| Median size, nm | 20 | 50 | 55 | 20 | 9.2 | 20 |
| Minimum size, nm | 5 | 12 | 20 | 3.3 | 6 | 3 |
| Average size, nm | 29.3 | 44 | 145.2 | 20 | 15.4 | 31.7 |

In contrast, the toxicity values of Ag NPs varied greatly: 275-fold for mammalian cells in vitro and 500-fold for bacteria. Assumingly, the differential toxicity of nanosilver was due to different coatings that were often applied on the surface of Ag nanoparticles to stabilize them. Indeed, all used ZnO and CuO NPs were uncoated (Tables S5 and S7) but 60 % of Ag NPs used in studies with bacterial cells and 89 % of Ag NPs used in studies with mammalian cells were coated (Table S3). In case of mammalian cells, 55 % of studied Ag NPs had PVP coating, 24 % had peptide coating, and 11 % was uncoated. In case of bacterial cells PVP, mono- and disaccharides and biogenic coatings were reported. Interestingly, the uncoated Ag NPs were remarkably less inhibitory to bacteria than coated NPs. Specifically, to various bacterial strains 14 least inhibitory Ag NPs (MIC values >17 mg/L) were all uncoated. Within 32 Ag NPs that were inhibitory to bacteria at lower than 14 mg/L concentrations 28 were coated and only 4 uncoated, whereas the type of the coating seemed to play no role (Table S3). In case of mammalian cells in vitro we did not observe analogous effect of coating (Table S3).

Finally, we analyzed the obtained toxicity data with respect to the size of NPs. Information on size of NPs for which mammalian cell and bacterial toxicity data (Tables S3, S5 and S7) were collected is shown in Table 2. The median sizes of Ag, CuO and ZnO were 20, 50 and 55 nm, respectively, for mammalian cells in vitro and 20, 9.2 and 20 nm, respectively, for bacterial cells.

Example on correlation between toxicity of Ag NPs to mammalian cells in vitro and the NPs primary size is given in Fig. 9a. To avoid the interference of coating in Ag NPs' toxicity, only PVP-coated NPs were used. When all the retrieved L(E)C50 values of PVP-coated Ag NPs to mammalian cells were plotted against the primary size on these NPs, no correlation was observed ($R^2 = 0.1$) (Fig. 9a). At the same time, higher correlation ($R^2 = 0.4$) was observed when the toxicity data from one single article was used (Liu et al. 2010). Finally, when the toxicity data

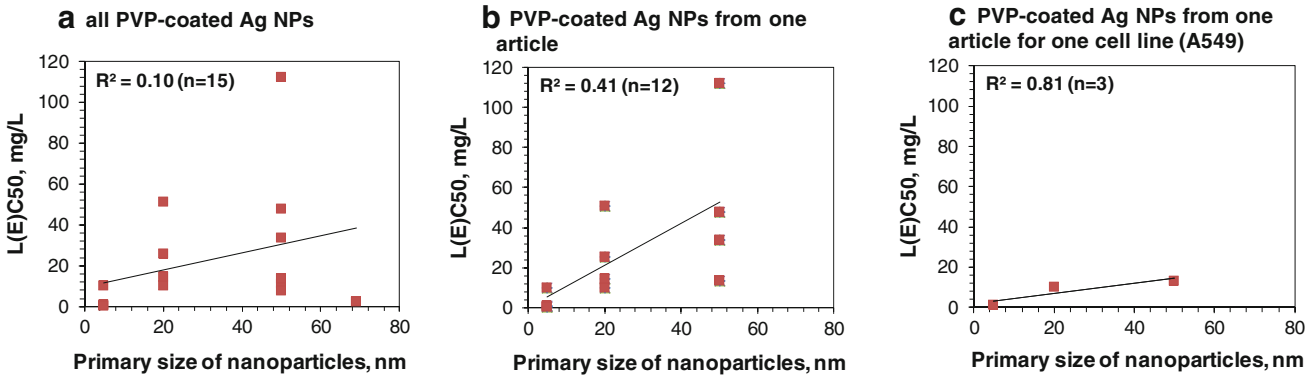


**Fig. 9** L(E)C50 values of PVP-coated Ag NPs to mammalian cells versus size of nanoparticles. **a** All collected data were used; **b** data from one article (Liu et al. 2010) were used; **c** data from one article for one cell type were used (Liu et al. 2010)

for one cell line from one article was used, clear correlation was observed between the size and the toxicity of NPs ($R^2 = 0.81$, Fig. 9c). Similar observations were done for other articles that presented the toxic effects of a library of differently sized well-characterized NPs for various organism groups (Martínez-Castanón et al. 2008; Hoheisel et al. 2012; Wang et al. 2012a). These findings show clearly that the interlaboratory variations in preparation of NP suspensions and toxicity testing conditions make it difficult to draw general conclusions regarding the toxicity of NPs. At a single laboratory level, this problem may be resolved by using well-characterized monodisperse libraries of NPs. At the level of the whole nanotoxicology community, it is very important to proceed with the implementation of the general guidelines for nanotoxicology research to end up with the parameters that should be addressed in every nanotoxicological work, for example sufficient characterization of NPs and utilization of technically suitable toxicity tests and reference materials (Nature Nanotech Editorial 2012).

## Conclusions

Our analysis of the literature data showed that:

1. The most toxic out of the three studied NPs was nanosilver. The L(E)C50 values of Ag NPs for the studied organisms/cells spanned nearly 4 orders of magnitude, from 0.01 mg/L for crustaceans to 38 mg/L for protozoa. For most of the species studied, the L(E)C50 values were below 10 mg/L, showing the hazardous properties of nanosilver compounds.
2. The $L(E)C_{50}$ values of CuO NPs ranged from 2 to 3 mg/L for crustaceans and algae, to >100 mg/L for protozoa and bacteria, and were in the range of 10–100 mg/L for most of the organisms studied.
3. ZnO NPs were the most toxic to algae (<0.1 mg/L), followed by crustaceans, fish, bacteria *V. fischeri* and protozoa. The L(E)C50 values of ZnO NPs were between 10 and 100 mg/L for nematodes, yeast and mammalian cells. Interestingly, ZnO NPs were not toxic to bacteria (median MIC 622 mg/L).
4. The toxic effect of Ag NPs and ZnO NPs (but not CuO NPs) was seemingly explained by solubilized ions. The intraspecies differences in toxicity seem to be at least partially explained by the composition of the test medium that affects the solubilization of metal-containing NPs and speciation of released metal ions.
5. Although bacterial cells are one of the target groups for all the studied nanoparticles, bacteria were among the least sensitive organisms. Instead, all the studied nanoparticles were remarkably more toxic to crustaceans, algae and fish.
6. Notably, one group of aquatic organisms most affected by the studied NPs was algae. This observation is noteworthy because planktonic microalgae as primary producers are the key component of food chain in aquatic ecosystems. Also, many algal species serve directly as a food source for zooplankton, which is subsequently consumed by other invertebrates or fish. Changes in the structure and productivity of the algal community may induce direct structural changes in the rest of the ecosystem and/or indirectly affect the ecosystem by affecting water quality (Nyholm and Petersen 1997).

## Outlook

Crustaceans, algae and fish—the aquatic test organisms proposed for the classification and labeling of chemicals by EU REACH regulation—proved the most sensitive groups of organisms with respect to the toxic action of all three analyzed metal-containing NPs. Unexpectedly, the analysis of the published data on toxic effects of Ag, ZnO and CuO NPs showed that these three biocidal NPs were inhibitory to bacteria at considerably higher level than to non-target environmental organisms. Our observation is coherent with the recent statement of Chernousova and Epple (2013) on nanosilver: 'After analyzing a multitude of single studies, it can be concluded that the effect of silver towards bacteria is typically overestimated, and towards (eukaryotic) cells it is typically underestimated. Therefore, the application of silver in consumer products, cosmetics, and medical products should be critically assessed.'

To address the environmental impact of biocidal nanomaterials, we would like additionally to emphasize the following aspect of the species sensitivity pattern toward nanomaterials: As the toxicity range for all the three metal-containing NPs to non-target aquatic organisms and target organisms (bacteria, fungi, algae) warningly overlapped, the discharge or leaching of biocidal nanomaterials to surface waters may pose threat to aquatic species. This aspect of life cycle of nanomaterials could be controlled either at the level of 'safe by design' or, if applicable, by regulated discharge/disposal.

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