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Review

From ecotoxicology to nanoecotoxicology

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ABSTRACT

For hazard assessment of NPs quantitative nanoecotoxicological data are required. The objective of this review was to evaluate the currently existing literature data on toxicity (L(E)C50 values) of synthetic NPs in environmentally relevant species in order to: (i) identify tentatively most harmful NPs and most sensitive organism groups, and (ii) to provide relevant ecotoxicological information for further risk assessment. The focus was set on selected synthetic NPs (nano TiO_2, nano ZnO, nano CuO, nano Ag, SWCNTs, MWCNs and C60-fullerenes) and organism groups representing main food-chain levels (bacteria, algae, crustaceans, ciliates, fish, yeasts and nematodes).

Altogether 77 effect values were found, mostly for nano TiO_2 (31%), C60 (18%), nano ZnO (17%), nano Ag (13%), SWCNTs and nano CuO (both 9%). Only 3% of the available quantitative ecotoxicological information concerned MWCNTs. Organism-wise, 33% of the data concerned crustaceans, 27% bacteria, 14% algae and 13% fish. For all organism groups studied, solubility of CuO- and ZnO-NPs was a key factor in their aquatic toxicity.

On the basis of the 34 median L(E)C50 values derived from 77 individual values, NPs were ranked according to their lowest median L(E)C50 value for the above described organism groups: the most harmful were nano Ag and nano ZnO that were classified "extremely toxic", (L(E)C50 < 0.1 mg/l), followed by C60 fullerenes and nano CuO that were classified "very toxic", (L(E)C50 0.1–1 mg/l). SWCNTs and MWCNTs were classified "toxic" (L(E)C50 1–10 mg/l). Nano TiO_2 was classified as "harmful", (L(E)C50 10–100 mg/l). Throughout, algae and crustaceans (daphnids) were most sensitive and thus probably most vulnerable organism groups in aquatic exposure to NPs. Very low L(E)C50 values should deserve thorough attention of environmental risk assessors for evaluation of the potential adverse effects of synthetic NPs on ecosystems. As the quantitative nanoecotoxicological data are still rare, further studies are needed.

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1. Introduction

Nanotechnology is a rapidly expanding and advancing field of research that has already yielded a variety of commercially available products including cosmetics, suntan lotions, paints, self-cleaning windows and stain-resistant clothing. According to conservative estimates (Project on Emerging Nanotechnologies, 2008) the number of consumer products on the market containing nanoparticles (NPs) or nanofibers now exceeds 800 and is growing rapidly. According to "The Nanotechnology Consumer Products Inventory" (Maynard and Michelson, 2006) the most common material mentioned in the product descriptions was carbon (29 products) which included fullerenes and nanotubes. Silver was the second most referenced (25 products), followed by silica (14), titanium dioxide (8), zinc oxide (8), and cerium oxide (1). Among potential environmental applications of NPs, remediation of contaminated groundwater with nanoscale iron is one of the most prominent examples (Zhang, 2003; Tratnyek and Johnson, 2006). Regarding personal-care products, NPs of titanium dioxide and zinc oxide are included in toothpaste, beauty products, sunscreens (Serpone et al., 2007) and textiles (Yuranova et al., 2007). In addition, silver NPs are increasingly used as antimicrobial additives in detergents, food packaging and textiles such as socks and underwear (Maynard and Michelson, 2006). Müller and Nowack (2008) have provided their best guess for the current worldwide production of nano TiO_2 5000 t/a, for nano Ag 500 t/a, and for carbon nanotubes 350 t/a. It has been reported that U.S. production of TiO_2 in 2005 was over 2 million tons and full conversion from bulk TiO_2 production to nano TiO_2 has been predicted by 2025 (Uyar et al., 2007). The above mentioned NPs are currently also most widely studied as reflected by the availability of data in various databases: most of the data concerns carbon-based synthetic NPs (carbon nanotubes, fullerenes), silver NPs and titanium dioxide NPs (Table 1).

Due to the increased production of synthetic NPs, the occupational and public exposure to NPs is supposed to increase dramatically in the coming years as well as their potential release in the environment. A pioneering study of Oberdörster (2004) showed that C60 fullerenes were inducing changes in the brain of the fish already at very low aquatic exposure level. Namely, significant lipid peroxidation was found in brains of largemouth bass after 48 h of exposure to 0.5 mg/l of uncoated C60 fullerenes (tetrahydrofuran was used for solubilisation of C60). In addition to the adverse effects on fish, the author also observed the improvement of aquaria water clarity with both 0.5 and 1 mg/l C60 suggesting that fullerenes may be bactericidal.

Three key elements of NPs toxicity screening strategies have been outlined by Oberdörster et al. (2005a): (i) physicochemical characterization (size, surface area, shape, solubility, aggregation), elucidation of biological effects involving (ii) *in vitro* and (iii) *in vivo* studies. These three key elements were formulated mainly from the point of view of potential effects of NPs on humans. When the whole ecosystem is concerned, the problem is more complicated. Although there is already remarkable amount of toxicological information concerning NPs available (obtained at various biological levels, starting from *in vitro* cell cultures and ending by *in vivo* studies on rodents), ecotoxicological data on NPs are just emerging. However, there is a remarkable amount of data and experience on environmental hazard evaluation of bulk chemicals. The challenge and task for nanoecotoxicologists is to analyze this information, critically evaluate and take the significant data and concepts on board, to synthesize new knowledge and approaches based on "old/existing" (dose–effect data, protocols, QSARs—quantitative-structure–activity-relationships) and "modern" knowledge that evolution of the science has introduced (toxicogenomics, biomarkers).

2. Nanotoxicological research and EU policy

Currently, assessing the safety of synthetic NPs has become a worldwide issue. The ecotoxicological research on NPs is also supported and promoted by EC science policy. On the 7th June 2005, the Action Plan "Nanosciences and nanotechnologies: An Action Plan for Europe 2005–2009" was adopted (European Commission, 2004) for the "immediate implementation of a safe, integrated and responsible strategy for nanosciences and nanotechnologies". In this document, the Commission undertook to ensure that appropriate research will be conducted to provide quantitative data on toxicology and ecotoxicology (including human and environmental dose response and exposure data), and to make sure that risk assessments are carried out. With the closing date 28.2.2006, Commission launched a consultation on nanotoxicology and nanoecotoxicology (European Commission, 2006) and asked stakeholders to identify priorities and potential actions. Contributions were expected to address several areas, including (i) research and development in nano(eco)toxicology, *i.e.* identification and prioritization of research on safety and risk assessment; (ii) generation of data on toxicology and ecotoxicology, and evaluation of potential human and environmental exposure; and (iii) need for databases on toxicity and to share toxicological, ecotoxicological and epidemiological data.

Originally, nanotechnologies and nanomaterials were not included in the scope of the Regulation (EC) No. 1907/2006 (REACH) (European Parliament, 2006). However, there are ongoing discussions within the REACH competent authorities and its subgroup on nanomaterials on how REACH applies to nanomaterials (CA/59/2008 rev. 1; Brussels, 16 December 2008; European Commission, 2008). In addition, the new EU Regulation 1272/2008 (European Parliament, 2008) on the classification, labeling and packaging of substances and mixtures that took effect on 20.01.2009 annulled the previous Dangerous Substances Directive 67/548/EEC and the Dangerous Preparations Directive 1999/45/EC. This new regulation is following the principles of the United Nations Globally Harmonised System (GHS) of Classification and Labeling of

Table 1
Availability of data for different types of nanoparticles and their potential hazardous effects in Science Direct, Thomson Scientific Web of Science and Google Scholar. Search was made in March 2009.

Keyword	Data on different nanoparticles (number of records) in various databases			Data on potential hazard of different nanoparticles (number of records in Science Direct, March 11, 2009, all fields)	
	Science Direct, search March 11, 2009	Web of Science (field "topic", all years. Search March 10, 2009	Google Scholar, Search March 10, 2009	AND[a] health	AND[a] hazard
Nanoparticles	56,894	83,295	490,000	5856	1693
Carbon nanotubes	21,929	33,782	151,000	1524	556
Fullerenes	11,366	12,032	100,000	662	197
Silver nanoparticles[b]	8,225	4,242	11,600	974	370
Nano TiO_2	694	579	4,430	107	104
Nano ZnO	198	147	1,490	20	15
Nano CuO	57	26	184	9	3
Nano Al_2O_3	193	141	859	6	2
Nano SiO_2	275	264	2430	10	9
Nano Fe_2O_3	24	21	60	3	0

[a] AND means that the keyword in the first column was combined either with the keyword "health" or "hazard".
[b] Search made in April 4, 2009.

Chemicals. Regulation 1272/2008 is a document of 1355 pages and does not contain term "nano" or "nanoparticle". However, as a general principle, it applies to all substances and mixtures supplied in the Community. Although there are no provisions in REACH referring specifically to nanomaterials, REACH deals with substances, in whatever size, shape or physical state. Thus, it follows that under REACH and the new Regulation 1272/2008 manufacturers, importers and downstream users have to ensure that their nanomaterials do not adversely affect human health or the environment.

3. From ecotoxicology to nanoecotoxicology

Ecotoxicology is a relatively new science concerned with contaminants in the biosphere and their effects on constituents of the biosphere, including humans (Newman and Zhao, 2008). The term ecotoxicology was coined by René Truhaut in 1969 who defined it as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut, 1977). Ecotoxicological research was rapidly developing due to the pollution of the environment induced by the rapid industrial development. Also, research was speeded up by severe industrial accidents (Seveso, Minamata, Exxon Valdez). Policies were developed accordingly and ecotoxicology became an important part in environmental and ecological risk assessment. Contrarily to the approaches driven by analytical chemistry, ecotoxicological tests integrate all toxic signals and thus, it has been proposed to add toxicity-based criteria to the currently existing policies for the meaningful evaluation of the environmental hazard (Manusadzianas et al., 2003; Põllumaa et al., 2004; Kahru and Põllumaa, 2006). The ecotoxicology developed mostly as aquatic toxicology and terrestrial ecotoxicological studies lag behind aquatic ones, as stated already 20 years ago by van Straalen and Denneman (1989). Blaise (1998) classified the aquatic toxicity research decade-wise till 1990s as follows: "the dark ages" in the 1950s and before; "the fish-testing 1960s"; "the regulatory 1970s"; "the ecotoxicological 1980s" and "the microbiotesting 1990s". A recent article by Bard (2008) in "Encyclopedia of Ecology" gives updated condensed information on the current state of the art of ecotoxicology. There are clear tendencies of development of both, terrestrial and aquatic ecotoxicology through the movement of traditional ecotoxicology into toxicogenomics (Spurgeon et al., 2008). Thus, in the same spirit as Blaise, the current review would designate the signature for the next decade(s) as "the (eco)toxicogenomical and nano(eco)toxicological 2010s".

Despite of a growing understanding that synthetic NPs should be evaluated for their potential environmental hazard *prior* their use in products and subsequent inevitable release into the environment, there are currently few data on the toxicity of nanomaterials to environmentally relevant species, limiting the quantitative risk assessment of NPs. Indeed, nanotoxicology research started in the early 1990s as shown by the first few scientific papers recorded in Web of Science of Thomson Scientific (formerly known as Thomson ISI) and this research was remarkably supported by the earlier studies concerning (pulmonary) effects of ultrafine particles (Oberdörster et al., 2005b). There was a lag of almost 10 years till number of nanotoxicological papers started to increase exponentially (Fig. 1). However, most of the data has been obtained on limited types of particles and mostly on *in vitro* cell cultures or *in vivo* respiratory exposures on rodents (The Royal Society, 2004). Despite of that there is already considerable amount of information available referring to human health (Table 1).

As previously in any risk or impact assessment of bulk chemicals, environmental concern of NPs appeared later and the first papers on nanoecotoxicology were published in 2006 (Fig. 1). Of course, the above statement cannot be taken literally as it is based on bibliometric indicators which always depend on the choice of the keywords. Indeed, one of the first papers where potential environmental hazards of nanotechnologies were shown clearly is the

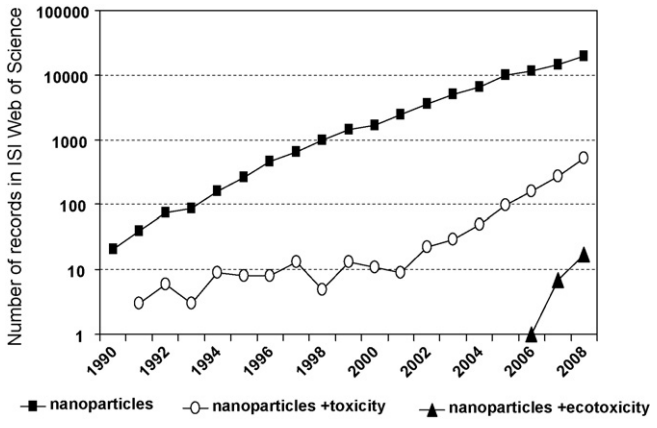

Fig. 1. Number of records in ISI Web of Science of Thomson Scientific. Search was made in April 3, 2009 using keywords "nanoparticles", "nanoparticles AND toxicity" and "nanoparticles AND ecotoxicity" (field: topic). "AND" means combination of the respective keywords.

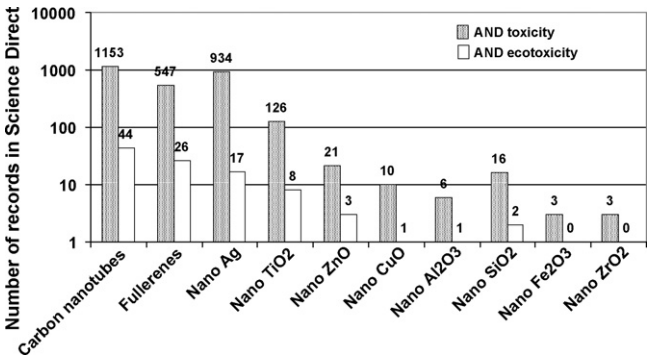

Fig. 2. Availability of data (number of records) concerning toxicological and ecotoxicological effects of various nanoparticles. Search was made in Science Direct; March 11, 2009 (all fields). For silver nanoparticles the search was made in April 4, 2009. "AND" means combination of the respective keywords.

paper by Colvin (2003) but the term "ecotoxicity" was not used in this paper. Also, the pioneering paper of Oberdörster on C60 effects on fish (see above) published already in 2004 was not using word "ecotoxicity".

It should be stressed that natural NPs, including nano-sized metal oxides, exist in all ecosystems and during evolution the living organisms have adapted to them. For synthetic NPs, however, their potential harmful properties on ecosystems have to be evaluated (Handy et al., 2008a) as the ecotoxicological data on NPs are just emerging (for reviews, see Baun et al., 2008a; Handy et al., 2008a; Kahru et al., 2008; Navarro et al., 2008a). According to Baun et al. (2008a) by 2008 there were less than 50 open peer-reviewed ecotoxicity scientific papers on environmentally relevant species. Currently, the investigation of the behavior and effects of NPs in the environment is no longer in its infancy but many investigations still have explorative character and raise more hypothesis than true answers (Nowack, 2009). As an indicator, a bibliometric search in Web of Science made in April 2009 using "nanoparticles AND toxicity" yielded 1381 records and "nanoparticles AND ecotoxicity" gave 31 records (Fig. 1). The highest number of records was obtained for fullerenes, carbon nanotubes, TiO_2 and silver-nanoparticles (Fig. 2).

4. Challenges in nanoecotoxicological research

The recent publication by Behra and Krug (2008) in "Nature Nanotechnology" section "News and Views" indicates three main problems that should be solved within the next few years: (i) the choice of nanoparticles to use in biological experiments, and the tests (analysis of physico-chemical properties, aggregation, sedimentation, etc.) needed to characterize them before, during and after these experiments, need to be determined; (ii) the need to examine the route of uptake of synthetic NPs by organisms in different environments (important for the behavior of synthetic NPs in the food-chain); (iii) the choice of organisms and endpoints measured.

The above mentioned challenges and what has been already done to solve these problems will be discussed below.

4.1. Representative manufactured nanomaterials for testing

In 2005, the International Life Sciences Institute Research Foundation/Risk Science Institute convened an expert working group to develop a screening strategy for the hazard identification of engineered nanomaterials. The expert groups outlined three key elements of the toxicity screening strategy: physicochemical characteristics, *in vitro* assays (cellular and non-cellular), and *in vivo* assays. In addition, as a "research gap", the development of standardized, well characterized nanomaterial samples was stated (Oberdörster et al., 2005a).

The Organization for Economic Co-operation and Development (OECD) Council established in 2006 the Working Party on Manufactured Nanomaterials (WPMN), to help member countries efficiently and effectively address the safety challenges of nanomaterials. In 2008, the WPMN published a report ENV/JM/MONO (2008)13/REV that defined a list of 14 representative manufactured nanomaterials for testing. In this report the word "representative" refers to those manufactured nanomaterials sooner or later to enter the market, for inclusion in a set of reference materials to support measurement, toxicology and risk assessment of nanomaterials. This list includes 8 inorganic nanomaterials: silver NPs, iron NPs, titanium dioxide, aluminum oxide, cerium oxide, zinc oxide, silicon dioxide, nanoclays and 6 organic nanomaterials: carbon black, fullerenes (C60), single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), polystyrene, dendrimers.

Concerning REACH, substances in nanoform which are in European Inventory of Existing Commercial chemical Substances, EINECS (*e.g.*, titanium dioxide) shall be regarded as existing substances and substances in nanoform which are not in EINECS (*e.g.* carbon allotropes other than those listed in EINECS) shall be regarded as new substances (European Chemicals Bureau, 2006).

4.2. Bioavailability of synthetic NPs in different environments

Ecotoxicological tests were mostly developed for aquatic test organisms and water-soluble chemical compounds. Thus, aquatic toxicity testing of NPs is definitely a challenge. However, whatever the apparent route of exposure and the mechanisms of toxicity, bioavailability remains a key factor for the hazard evaluation of synthetic NPs. Bioavailability is a dynamic concept that considers physical, chemical, and biological processes of contaminant exposure and dose. Bioavailability incorporates concepts of environmental chemistry and ecotoxicology, integrating contaminant concentration, fate, and an organism's behavior in the given environment. Bioavailability of NPs depends on the: (i) physico-chemical properties of the particles (aggregation, solubility), (ii) on nanoparticle-organism contact environment, but also (iii) on the target organism (particle-ingesting or not). Thus, environmental risk assessment of synthetic NPs requires thorough characterization of NPs before, during and after exposure. Many methods still need development and optimization, especially for new types of NPs, but extensive experience can be gained from environmental chemistry.

4.2.1. Physico-chemical properties determining the bioavailability and toxicity of NPs

It is well known that at nanosize range, the properties of materials differ substantially from bulk materials of the same composition, mostly due to the increased specific surface area and reactivity, which may lead to increased bioavailability and toxicity (Nel et al., 2006). Indeed, NPs of CuO were up to 50-fold more toxic than particles of bulk CuO towards crustaceans (Heinlaan et al., 2008), algae (Aruoja et al., 2009), protozoa (Mortimer et al., 2010) and yeast (Kasemets et al., 2009). TiO_2 and Al_2O_3 NPs were about twice more toxic than their respective bulk formulations towards nematodes (Wang et al., 2009). However, none of the above mentioned authors did observe significant differences in toxicity of nano and bulk ZnO and that will be discussed below. Concerning silver NPs, their antibacterial effect has been shown to depend not only on size (Morones et al., 2005) but also on shape (Pal et al., 2007). Inhibitory effect of silver NPs to nitrifying organisms correlated with the fraction less than 5 nm, as NPs of these sizes were more toxic to bacteria than any other fractions of NPs or their bulk species (Choi and Hu, 2008).

The surface properties of NPs are also one of the most important factors that influence their stability and mobility as colloidal suspensions or their aggregation into larger particles and deposition in aquatic systems (Navarro et al., 2008a). As in aquatic systems significant sedimentation of NP aggregates can be expected, sediments should be regarded as an important sink for synthetic NPs discharged to the aquatic environment (Baun et al., 2008a) and benthic organisms as key receptors for NPs (Christian et al., 2008). Indeed, in aqueous media NPs often form visible aggregates, leaving only a minor part of the total mass of NPs in the size ranges relevant for direct uptake throughout the water phase. It has thus been suggested (Baveye and Laba, 2008) that aggregation may have implications on toxicity, which may result in very dissimilar biological activity. For example, entrapping of algal cells in aggregates of nano TiO_2 may play a major role in toxicity to algae *Pseudokirchneriella subcapitata* (Aruoja et al., 2009).

The solubilisation of metal-containing NPs that is compound-, test media-, temperature- and time-dependent can be a key factor in their toxicity (Brunner et al., 2006; Franklin et al., 2007; Kahru et al., 2008; Wang et al., 2009). Indeed, as shown for metal-oxide NPs (incl. TiO_2 and ZnO) using *in vitro* cell cultures, solubility of those NPs strongly influenced their cytotoxicity (Brunner et al., 2006). The bactericidal effect of silver NPs was also due to the release of silver ions (Morones et al., 2005; Rai et al., 2009). Thus, the test results of relatively soluble metal-containing NPs will depend also on complexing of the solubilised metals depending mostly on test environment used (Witters, 1998) and on organism–NP interactions (Navarro et al., 2008b). Indeed, metal solubility may be changed by organisms: initially insoluble forms of heavy metals may become bioavailable due to the direct contact between bacteria and soil particles (Kahru et al., 2005a).

The impact of solubilisation of metals from metal-containing NPs may be studied in a combined approach involving chemical analysis and recombinant metal-specific microbial sensors. Those genetically modified microbial biosensor strains will only produce a response if the toxic compound (for example, heavy metal) crosses the cell biological envelopes and enters the cytoplasmic space, that is if the toxicant is accessible or bioavailable to the sensing system (Ivask et al., 2009). This approach has been successfully used for the demonstration of the toxic effect of solubilised Zn (using Zn-sensing bacteria) and Cu (using Cu-sensing yeast and bacteria) from NPs of ZnO and CuO, respectively towards algae (Aruoja et al., 2009), crustaceans and bacteria (Heinlaan et al., 2008), protozoa (Mortimer et al., 2010) and yeast (Kasemets et al., 2009), *i.e.*, for both the particle-feeding and particle-"resistant" organisms. It has been stressed that water 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).

4.2.2. Modulation of bioavailability by environmental factors

As discussed above, the environmental fate of synthetic NPs and thus the bioavailability of the toxic "component" will also depend on the interactions of synthetic NPs with aquatic colloids that may strongly influence their behavior in surface waters (Lead and Wilkinson, 2006; Klaine et al., 2008). Indeed, in natural aquatic systems, sparingly soluble contaminants are predominantly bound to particle surfaces or complexed by, *i.e.*, humic substances. Due to their high surface to mass ratio, natural NPs play an important role in the solid/water partitioning of contaminants (Christian et al., 2008). For example, humic substances in natural waters often mitigate the toxic effects of pollutants (especially that of toxic heavy metals) to aquatic organisms (Kramer et al., 2004). Analogously to humic substances, synthetic NPs have been demonstrated to sorb pollutants: Baun et al. (2008b) showed that the toxicity of phenanthrene for *Daphnia magna* was increased by 60% in the presence of C60 aggregates and that sorbed phenanthrene was available for the organisms. Thus NPs not only act as transfer vectors in the environment, but they also facilitate the entry of NP-sorbed pollutants into cells/organisms potentiating toxic effects. Several reports and reviews on NP safety state that there are knowledge gaps concerning the ability of NPs to act as vectors of chemicals, microorganisms and interactions with other stressors (Moore, 2006; The Royal Society, 2004). However, to date, there have been few systematic ecotoxicological studies investigating how the changes in abiotic factors such as pH, salinity, hardness, ionic strength, or the presence of organic ligands in the water influence ecotoxicity of NPs (Handy et al., 2008a; Blinova et al., 2009). Lastly, it is well known that the water column in lakes and marshes is heterogeneous with variations not only in pH, but also in dissolved oxygen and redox potential (Zehnder, 1988). Thus research on the fate of NPs in the aquatic environment should take in account all concepts of biogeochemistry and limnology, particularly when addressing anoxic layers and bottom sediments.

4.2.3. Biotic factors determining the bioavailability of NPs

One may assume that unicellular organisms *a priori* not internalizing particles (bacteria, algae) should be more resistant to toxic effects of NPs than protists and metazoans that have highly developed systems for internalization of nano and micro-scale particles. However, as discussed above, this is not straightforward as some NPs (CuO, ZnO) are quite soluble and cause toxicity by solubilised ions (Cu^{2+}, Zn^{2+}) (Kahru et al., 2008) and synthetic NPs also cause extracellular toxic effects. For example, toxicity of TiO_2 NPs may involve generation of hydroxyl radicals due to visible light generating extracellular reactive oxygen species (ROS) that may damage cell membranes. Indeed, TiO_2 NPs in combination with UV light (370 nm) have been shown to inactivate algae (Kim and Lee, 2005). Analogously, in bacteria which have a rigid cell wall and cytoplasmic membranes, NPs can disrupt these barriers by the dissolved ions and/or oxidative stress, changing the membrane permeability and increase the probability of entry of NPs into the cell. This has been shown for ZnO NPs on Gram-positive bacteria *Streptococcus agalactiae* and *Staphylococcus aureus* (Huang et al., 2008). Silver NPs (<10 nm) have shown to get attached to Gram-negative *Escherichia coli* cell wall (due to the presence of thiol groups in cell wall proteins), resulting in the perforation of the cell wall, and leading to the cell death (Gogoi et al., 2006). The antimicrobial mechanism of silver NPs may also be related to membrane damage due to free radicals derived from the surface of the NPs (Kim et al., 2007). These mechanisms could explain the considerable numbers of silver NPs found inside the bacteria *E. coli* by Morones et al. (2005). Also, <5 nm CdSe and CdSe/ZnS quantum dots have been shown to enter the bacteria *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative) probably by oxidative damage of the cell membrane potentialised by light (Kloepfer et al., 2005).

In particle-ingesting organisms, accumulation of NPs in the digestive tract may be observed in *Daphnia* and other organisms (Baun et al., 2008a; Kahru et al., 2008). However, uptake of NPs *via* digestive tract cells remains questionable at least in the case of *Daphnia* (Heinlaan et al., 2009) although the toxic influence of NP-sorbed pollutants is a very realistic exposure scenario (Baun et al., 2008b). Kashiwada (2006) used water-suspended fluorescent NPs (solid latex solution) to investigate the distribution of NPs in the eggs and bodies of the see-through medaka (*Oryzias latipes*). Adult medaka accumulated 39.4-nm NPs mainly in the gills and intestine when exposed to a 10-mg/l NP solution for 7 days (no mortality during the exposure). NPs were also detected in the brain, testis, liver, and blood. The gills, a priority organ in its contact with xenobiotics, showed the most significant accumulation of NPs. The NPs entered the circulation through the membranes of the gills and/or the intestine, and evidence of olfactory neuron migration of particles was

not found. Differently, Oberdörster (2004) proposed that exposure of the brain of largemouth bass to C60 fullerenes could occur *via* olfactory neurons. Comparing NPs of TiO_2 and C60 fullerenes Handy et al. (2008b) concluded that the uptake mechanisms for NPs by epithelial cells are more likely to occur via vesicular processes (*e.g.*, endocytosis) than uptake on membrane transporters or by diffusion through the cell membranes. Target organs may include the gills, gut, liver and sometimes the brain. In embryos of zebrafish, Harper et al. (2007) showed that biodistribution was influenced by chemical composition of NPs as well as route of exposure. Thus, FluoSpheres® administered via the oral route of exposure were retained within the gastrointestinal tract whereas Qdots® were readily taken up across the gastrointestinal tract and distributed to the brain. Uptake from a dermal route was primarily limited to the epithelial layers and the yolk sac for carboxylated FluoSpheres®, but distribution to the brain region was achieved from waterborne exposure to Qdots®. The authors also stress that immense data gaps and conflicting reports on nanotoxicology currently prevent generalizing how NP physicochemical properties relate to biological activity and toxic potential and conclude that *in vivo* animal models, such as the zebrafish, are needed to interpret the effects of nanomaterial exposure in a whole animal context.

In mammalian cells *in vitro*, uptake of NPs has been clearly shown for various cell types. This uptake may proceed *via* different processes, including endocytosis and phagocytosis. Human Caco-2 cells have been developed as a useful alternative to animal models to study intestinal absorption of various compounds. Therefore, the NPs uptake studies observed in Caco-2 cells could probably be considered to correlate with *in vivo* situations. Lai et al. (2008) showed only limited uptake of water-insoluble NPs (charcoal NPs, pyrene NPs) in Caco-2 cells differently from the murine macrophage-like cells, RAW, where the uptake was significant. In addition, the interplay of the pH and chemical composition in the organism's gut or in some other relevant exposure compartment may change the speciation and thus toxicity. For example, in human lung epithelial cells *in vitro*, Limbach et al. (2007) have shown that partially soluble NPs such as cobalt oxide and manganese oxide may be taken up into cells by a Trojan-horse type mechanism, *i.e.*, metal-oxide NPs entered the cells but not the respective ionic forms. Once in the cell, these NPs may dissolve releasing higher damaging concentrations of metal ions within the cell.

4.3. The choice of organisms and relevant endpoints. Test batteries/suites

While risk assessment for human health concerns one species, environmental risk assessment (ERA) should ideally consider millions of species, with different morphology, physiology, and ecological conditions. For this reason, the current ecotoxicological testing, where a few species are representatives for such diverse groups as, *e.g.*, crustaceans, is of course a gross simplification of an ecosystem as well as a shortcoming in ERA (OECD, 1998). Indeed, as no single test or species of living organism show uniform sensitivity to all chemical compounds, a battery of biotests with different sensitivity profiles is often recommended and used to assure adequate evaluation of the ecotoxicological situation. Due to the complexity of ecosystems the ecotoxicological hazard assessment is more informative/predictive if the battery involves organisms of different trophic levels (Blaise, 1998; Blinova, 2000; Kahru et al., 2000, 2008; Manusadzianas et al., 2003; Kahru and Põllumaa, 2006).

For the regulatory decision-making, individual tests can be described in terms of cost, ecological relevance (validity), reliability (reproducibility), and sensitivity. There is often a trade-off between these properties. It is recommended to combine different tests so that the characteristics of the individual tests supplement each other (Breitholtz et al., 2006). For example, in regulatory testing of ecotoxicological hazard of pure chemicals, the following test species of different food-web level are recommended: fish (OECD Guideline 203), *Daphnia* (OECD Guidelines 202, 211), algae (OECD Guideline 201). These aquatic species are also often used for monitoring of water quality and hazard assessment of wastewaters since they respond in a predictable manner to the presence of most types of pollutants. As in the ecotoxicology the main vertebrate test organism has been fish, it is widely agreed that all information on a substance relevant to its potential acute aquatic toxicity should be evaluated *prior* to considering testing in fish, to avoid unnecessary use of animals whenever possible as well as reduce unnecessary testing costs. Baun et al. (2008a) stress that further aquatic invertebrate testing (especially long-term low exposure with chronic endpoints and bioaccumulation studies) will be important in ecotoxicological research of NPs. A recent review by Crane et al. (2008) show that the types of test species and biological endpoints used within standard environmental hazard assessment frameworks are generally appropriate also for nanoecotoxicological research. Furthermore, Kahru et al. (2008) and Blaise et al. (2008) stressed the importance of using the test battery concept developed for bulk chemicals for hazard evaluation of nanomaterials. Behind that concept, there is a knowledge accumulated during several decades of scientific and applied research.

5. Tools for nanoecotoxicological risk evaluation

It is obvious that all the problems concerning hazard evaluation of NPs cannot be rapidly solved. According to the tendencies of "growth curves" of nanotoxicological literature in Fig. 1, one may expect about 10,000 publicly available (new) ecotoxicological papers on NPs registered in Web of Science by about 2015. To speed up acquisition of new knowledge on nanoecotoxicology, or make this process more cost-efficient, one should learn from the knowledge that has already been collected involving not only time, money and human resources but also lives of experimental animals.

5.1. Old published data revisited

The knowledge on chemical constituents of nanomaterials, literature on their physico-chemical properties, mode of action, organism physiology, protocols for analysis and toxicity testing—this is all very valuable knowledge that one should take "on board", to move more rapidly in this highly competitive area of research. But, the physico-chemical behavior of the relevant environmental compartments should also be included. Indeed, for bulk chemicals, there exists a wealth of knowledge accumulated with decades of regulatory and scientific research on hazardous effects of chemicals and even more of this type of information will be needed due to the implementation of the REACH Directive. Due to the creation and rapid development of databases such as Science Direct, Thomson Scientific Web of Science and powerful search engines such as Google (Scholar), and increasing digitalization of old documents in these databases, the revisiting and dissemination of earlier obtained knowledge will be remarkably facilitated. In addition, revisiting of old (previously obtained) toxicological data are supporting the 3R's strategy that encourages reducing the number of experimental animals for toxicological research. Thus, it is very reasonable that the Annexes VII-X of the REACH directive (European Parliament, 2006) state: "all available *in vitro* data, *in vivo* data, historical human data, data from valid (Q)SARs and data from structurally related substances (read-across approach) shall be assessed first." The 3R's strategy is supported also by screening of chemicals/compounds for the toxicity using as a 1st tier the tests with non-vertebrate ecotoxicological organisms (cheap, medium- or high throughput, more ethical). We have shown that at the pre-

screening stage of all areas of *in vitro* toxicological research (not only in ecotoxicological studies) even as simple organisms as bacteria can be successfully used for various chemicals (Kahru, 2006) as well as for synthetic NPs (Kahru et al., 2005b).

5.2. Standardized ecotoxicological test protocols and tests developed for problematic chemicals/environmental samples

There exists a huge experience on testing and developing of standardized protocols (ISO, OECD, DIN) for bulk chemicals that have been worked out involving teams of most competent researchers. Of course, these testing protocols cannot be followed blindly, as NPs differ from bulk chemicals and thus practical information for handling poorly soluble chemicals and turbid or colored chemicals and/or environmental samples, may become helpful (OECD, 2000). Also, assays initially designed for turbid environmental samples such as suspensions of soils and sediments, may find use in nanotoxicological research. For example, a solid-phase flash assay—a kinetic *Vibrio fischeri* luminescence inhibition test (Lappalainen et al., 2001) that has been a very useful tool in soil and sediment toxicity testing (Põllumaa et al., 2000) has been successfully used for the screening of the ecotoxicity of turbid solutions of NPs (Mortimer et al., 2008; Heinlaan et al., 2008). Also, learning from environmental NPs (*e.g.*, humic colloids) on aggregation/disaggregation and sorption of pollutants may serve helpful (Nowack, 2009). Interactions of NPs with natural organic matter have to be considered as well, as those will alter the NPs aggregation behavior in surface waters or in soils (Navarro et al., 2008a; Blinova et al., 2009).

5.3. Modern techniques: (eco)toxicogenomics

Before manifestation of acute toxic effects on cell or organism level, the initial changes appear on molecular level. Thus, the elucidation of toxicant-specific molecular responses provides information on toxicity pathways already at subtoxic exposure levels. Due to the rapid development of "omics", the knowledge of toxicity pathways that is derived from genomic data would highlight the potential mechanisms and thus reduce the testing time and costs but also the need for experimental animals. As the number of entirely or partially elucidated genomes for ecologically relevant organisms (zebrafish, *Caenorhabditis elegans*, rainbow trout, Japanese medaka, fathead minnow, *Daphnia pulex*, *Xenopus* sp., *Tetrahymena thermophila*) is ever-increasing (Ankley et al., 2006; Eisen et al., 2006) and a significant parallel exists between genomic data and the effects endpoints, toxicogenomic methods will be soon more widely introduced for elucidation of toxicity pathways (Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council, 2007).

There is general agreement that toxicogenomics will also play an increasingly larger role in regulatory decision-making. The challenge is to comprehensively integrate the disparate chemical, biological, toxicological, and toxicogenomic data in order to elucidate the mechanisms and networks involved in toxicity and to develop quantitative models capable of accurately predicting thresholds (Boverhof and Zacharewski, 2006). Knowledge about mechanisms or mode of action is also fundamental to improve the scientific basis of environmental/ecological risk assessment (Escher and Hermens, 2002) and the term "ecotoxicogenomics" has recently also been proposed to describe the integration of genomics and ecotoxicology (Snape et al., 2004). Indeed, gene expression profiling – (eco)toxicogenomics – is a novel technique where sets of responsive genes of a cell (*in vitro*) or organism (*in vivo*) can be considered as toxicological endpoints. One of the most promising applications involves the screening and prioritization of commerce chemicals and drug candidates that warrant further development and testing. This consists of comparing their toxicogenomic profiles to databases containing profiles of known toxicants and identifying biomarkers of exposure and toxicity that can be used in high-throughput screening programs. Recently, the Comparative Toxicogenomics Database (CTD; http://ctd.mdibl.org/) has been created, to elucidate molecular mechanisms by which environmental chemicals affect human disease (Mattingly et al., 2006). CTD presents scientifically reviewed and curated information on chemicals, relevant genes and proteins, and their interactions in vertebrates (human, rat, mice, fish) and also in invertebrates (drosophila, nematode *C. elegans*, daphnids). Diverse model organisms and their sequence data can provide key insights into the molecular mechanisms of action of environmental chemicals and cross-species comparisons of toxicologically important genes present opportunities for associating sequence variation with functional differences. Currently CTD integrates toxicogenomic data for vertebrates and invertebrates, including 59,000 chemicals, 1.2 million gene and protein sequences (and their associated Gene Ontology and KEGG – Kyoto Encyclopedia of Genes and Genomes – pathway annotations), 83,000 taxonomic terms, and 6000 human diseases to produce a unique resource for the cross-species analysis of chemical, gene, and disease interactions (Wikipedia, 2009). Thus, although this database was created with the focus on humans, the genomic data deposited in this database will be informative also for ecotoxicological studies.

Microarrays have been used increasingly in ecotoxicological studies to determine the molecular mode of action of environmental pollutants and to identify their biomarkers as indicators of exposure and effect for risk assessments (Robbens et al., 2007). Although this technology is not as advanced in aquatic toxicology as it is for mammalian models, it has shown promise for developing "signatures" of chemicals that can be used for field and mixture studies. A major difficulty for the use of microarrays in aquatic toxicology is the lack of sequence information for non-model species. Also, recent studies that show non-linear toxic responses for ecological species highlight the necessity of establishing time and dose dependence of effects on gene expression and comparing these results with traditional markers of toxicity (Denslow et al., 2007). As the genomes of several environmentally relevant organisms are already sequenced, whole genome microarrays can be also used to define the transcriptional response to different types of NPs in order to assess the mechanistic basis of NP toxicity. In addition, the expression of selected genes can be confirmed by qPCR and visualized *in vivo* using various reporters (green fluorescence protein (GFP), luciferase) (Robbens et al., 2007). First data in this area are just emerging. For example, Zhu et al. (2006) have shown that tetrahydrofuran (THF)-nC60 was more toxic than water-stirred-nC60 in both daphnia and fathead minnow. Indeed, Henry et al. (2007) studied differentially expressed genes identified by microarray analyses of larval zebrafish and showed that degradation product of THF (used often as a solubilisation vehicle for C60 fullerenes) rather than C60 may explain toxicity attributed to C60. Thus, it is critical to use environmentally relevant doses and preparation techniques for ecotoxicological studies of NPs.

Despite of the challenges involved, the successful incorporation of toxicogenomics into regulatory frameworks for environmental risk assessment may someday be regarded as the most important intellectual and practical contribution from the current generation of ecotoxicologists (Ankley et al., 2006). And we may soon expect a new "omics" term—nanoecotoxicogenomics.

6. Current nanoecotoxicological knowledge

To start to fill the gap in nanoecotoxicological data, this part of the review will summarize current existing quantitative eco-

toxicological data for commercially available NPs, focusing mainly on aquatic organisms representing main food-web levels (bacteria, algae, crustaceans, ciliates and fish). In addition, data for the nematodes *C. elegans* that are abundant in soil ecosystems and play a key role in nutrient cycling, and data for yeast (*Saccharomyces cerevisiae*) are presented. The latter data were chosen for the comparison but also for providing the toxicological information for the potential toxicogenomic studies as genomes of these organisms are sequenced (Goffeau et al., 1996; The *C. elegans* Sequencing Consortium, 1998). Synthetic NPs important in environmental risk assessment context were chosen and this selection was expectedly coherent with the OECD list (see above). Data were collected for 3 organic NPs: C60 fullerenes, single-wall carbon nanotubes (SWCNT) and multiwall carbon nanotubes (MWCNT) as well as for 4 inorganic NPs: TiO_2, ZnO, CuO and nano Ag. For assessing the importance of the "nano" size and of the solubilisation of NPs, data for respective bulk metal oxides preparations and soluble salts of copper and zinc were also collected. Lastly, the data for two well known toxic chemicals – aniline and pentachlorophenol – were added as references as for these chemicals many L(E)C50 values for environmentally relevant species exist in various ecotoxicological databases. The comparison of the toxicity data of NPs with reference chemicals allows "to place" NP toxicities in a known scale.

6.1. Currently available quantitative nanoecotoxicological data

There is remarkable amount of ecotoxicological publications where the harmful properties of chemicals (*e.g.*, NPs) are discussed in a vague way. For example, some papers claim that a chemical/compound/nanomaterial is toxic or even very toxic without indicating (or bearing in mind) the concentration/dose showing these adverse effects. Thus, for a meaningful (eco)toxicological profiling of NPs, quantitative toxicity data (EC50, EC20, NOEC, LOEC, MIC) are required. EC50 values are the most precise values to estimate on a concentration–response curve (Isnard et al., 2001) and median EC50 values are usually used for the QAAR (quantitative-activity–activity-relationship) and QSAR analysis (Cronin et al., 2003).

For the current review, the L(E)C50 values for NPs, corresponding bulk formulations (if existing) and reference chemicals for environmentally relevant species were collected. In few cases also NOEC (no-observed-effect-concentration) or other endpoints values (such as minimum inhibitory concentration, MIC, or minimum killing concentration, MKC) were searched. These detailed data are presented in Supporting information of this paper (Tables S1–S6) including median, average, minimum and maximum values of L(E)C50 values for a certain compound and organism group as well as corresponding literature references. These data are summarized in Tables 2 and 3 as median L(E)C50 values (weight/volume in compound basis and, in case of soluble metal salts, on the basis of metal ion).

Concerning NPs, in total 77 effect values were collected for 7 NPs and 7 organism groups (Tables S1–S6) to end up with 34 median (or, if only two data were available, average) L(E)C50 values presented in Table 2. Most of the currently available quantitative nanoecotoxicological data concerns crustaceans (33%), followed by bacteria (27%), algae (14%) and fish (13%). There were only few data for ciliates, yeasts and nematodes (Fig. 3A). The distribution of data between organism-groups was practically similar for inorganic and organic NPs (data not shown). Particle-wise, most of the information concerned nano TiO_2 (31%), followed by C60 (18%), nano ZnO (17%), nano Ag (13%), SWCNTs and nano CuO (both 9%) and only 3% of the available quantitative ecotoxicological information concerned MWCNTs (Fig. 3B).

In addition to NPs, toxicity data were searched also for 3 bulk metal-oxide particles (ZnO, TiO_2, CuO) and 4 other reference chemicals (soluble Cu-salts and Zn-salts, pentachlorophenol and aniline). For the latter chemicals altogether 85 effect values (Tables S1–S6 available in the Supporting information) were summarized and their median L(E)C50 values are presented in Table 3.

6.2. Variability of L(E)C50 values within the same organism group

Fig. 4 shows that the data even for the same type of NP and group of organisms considerably varied. For example, bacterial toxicity data of nano Ag (various Gram-negative and Gram-positive bacteria, 5 values; Table S2—Supporting information) varied almost 3 orders of magnitude (Fig. 4A). Algal toxicity data for nano TiO_2 varied about 10-times (*P. subcapitata* and *Desmodesmus subspicatus*, 4 values; Table S3—Supporting information and Fig. 4B) that

Table 2
Median L(E)C50 values for selected synthetic nanoparticles towards different groups of organisms and classification of nanoparticles to different hazard categories. The classification scheme applied adheres to EU-Directive 93/67/EEC classification scheme (CEC, 1996). Evaluation grid applied by Sanderson et al. (2003) and Blaise et al. (2008) was used. Classification is based on median L(E)C50 value of the most sensitive organism used: <0.1 mg/l = extremely toxic to aquatic organisms; 0.1–1 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 = non-toxic to aquatic organisms. Lowest median L(E)C50 value for each compound is in bold. In the brackets the number of values that was used for the calculation of the median value is indicated. Data are summarized from Tables S1–S6 available in Supporting information.

No.	Group of organisms	Inorganic nanoparticles				Organic nanoparticles			Most toxic NP	No. of records[a]	Supporting information
		mg TiO_2/l Nano TiO_2	mg ZnO/l Nano ZnO	mg CuO/l Nano CuO	mg Ag/l Nano Ag	mg/l SWCNT	mg/l MWCNT	mg/l C60			
1	Crustaceans	67.7 (10)	0.62 (3)	2.65 (2)	**0.040** (1)	15.0 (3)	**8.7** (1)	35.0 (5)	Nano Ag	16 + 9 = 25	Table S1
2	Bacteria	603 (4)	20 (3)	71 (2)	7.60 (5)	163 (2)	500 (1)	0.81 (4)	C60	14 + 7 = 21	Table S2
3	Algae	**65.5** (4)	**0.068** (2)	**0.87** (1)	0.23 (2)	**1.04** (1)	NF	100.0 (1)	Nano ZnO	9 + 2 = 11	Table S3
4	Fish	300 (4)	1.9 (2)	NF	7.1 (1)	NF	NF	1.0 (3)	C60	7 + 3 = 10	Table S4
5	Ciliates	NF	5.4 (1)	156.5 (1)	39.0 (1)	6.8 (1)	NF	**0.25** (1)	C60	3 + 2 = 5	Table S5
6	Nematodes	80.1 (1)	2.24 (1)	NF	NF	NF	NF	NF	Nano ZnO	2 + 0 = 2	Table S6
7	Yeasts	20000 (1)	121.2 (1)	20.5 (1)	NF	NF	NF	NF	Nano CuO	3 + 0 = 3	Table S6
1-7	No. of data	24	13	7	10	7	2	14		54 + 23 = 77	
1-7	Lowest L(E)C50	**65.5**	**0.068**	**0.87**	**0.040**	**1.04**	**8.7**	**0.25**			
1-7	Most sensitive organisms	Algae	Algae	Algae	Crustaceans	Algae	Crustaceans	Ciliates			
1-7	Classification (1-7)[b]	Harmful	Extremely toxic	Very toxic	Extremely toxic	Toxic	Toxic	Very toxic			
1-3	Classification (1-3)[c]	Harmful	Extremely toxic	Very toxic	Extremely toxic	Toxic	Toxic	Very toxic			

NF, Not found.

[a] Used for the calculation of the median values (inorganic NPs + organic NPs = total).

[b] Classification of the potential hazard according to the lowest median L(E)C50 values (all test organisms).

[c] Classification of the potential hazard according to the lowest median L(E)C50 values (crustaceans, bacteria and algae).

Table 3
Median L(E)C50 values for bulk materials of ZnO, TiO_2 and CuO as well as for Zn^{2+}, Cu^{2+}, pentachlorophenol (PCP) and aniline towards different groups of organisms and their classification to different hazard categories. The classification scheme adheres to EU-Directive 93/67/EEC classification scheme (CEC, 1996). Evaluation grid applied by Sanderson et al. (2003) and Blaise et al. (2008) was used. Classification is based on median L(E)C50 value of the most sensitive organism used: <0.1 mg/l = extremely toxic to aquatic organisms; 0.1–1 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 = non-toxic to aquatic organisms. Lowest median L(E)C50 value for each compound is in bold. In the brackets the number of values that was used for the calculation of the median value is indicated. Data are summarized from Tables S1–S6 available in Supporting information.

No.	Group of organisms	Reference compounds							Most toxic chemical	Supporting information
		Bulk metal oxides			Metal ions		Organic chemicals			
		mg TiO_2/l Bulk TiO_2	mg ZnO/l Bulk ZnO	mg CuO/l Bulk CuO	mg Zn^{2+}/l Zn^{2+}	mg Cu^{2+}/l Cu^{2+}	mg/l PCP	mg/l Aniline		
1	Crustaceans	20000 (3)	0.48 (3)	127.8 (2)	0.192 (4)	0.029 (8)	0.48 (3)	**0.30** (3)	Cu^{2+}	Table S1
2	Bacteria	20000 (1)	20.0 (3)	3758 (1)	3.50 (5)	0.47 (4)	2.9 (4)	135 (2)	Cu^{2+}	Table S2
3	Algae	**60** (1)	**0.052** (2)	**14.2** (1)	**0.051** (2)	**0.020 (3)**	0.24 (1)	11 (3)	Cu^{2+}	Table S3
4	Fish	500 (2)	1.8 (2)	NF	36.9 (1)	0.21 (13)	0.23 (5)	49.0 (3)	Cu^{2+}	Table S4
5	Ciliates	NF	4.9 (1)	1947 (1)	9.8 (4)	1.10 (5)	**0.15** (1)	220 (2)	PCP	Table S5
6	Nematodes	137 (1)	2.2 (1)	NF	111.0 (3)	45.2 (2)	NF[a]	NF[a]	Bulk ZnO	Table S6
7	Yeasts	20000 (1)	134.4 (1)	1277 (1)	62.6 (2)	8.2 (2)	NF[a]	NF[a]	Cu^{2+}	Table S6
1-7	No. of data	9	13	6	21	37	14	13		
1-7	Lowest L(E)C50	**60**	**0.052**	**14.2**	**0.051**	**0.020**	**0.15**	**0.30**		
1-7	Most sensitive organisms	Algae	Algae	Algae	Algae	Algae	Ciliates	Crustaceans		
1-7	Classification (1-7)[b]	Harmful	Extremely toxic	Harmful	Extremely toxic	Extremely toxic	Very toxic	Very toxic		
1-3	Classification (1-3)[c]	Harmful	Extremely toxic	Harmful	Extremely toxic	Extremely toxic	Very toxic	Very toxic		
	Risk phrases (R)[d]	NF	R50/R53	NF	R50/R53	R50/R53	R50/R53	R50		
	Hazard class and category code (hazard statement code)[e]	NF	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410)	NF	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410)	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410)	Aquatic Acute 1 (H400); Aquatic Chronic 1 (H410)	Aquatic Acute 1 (H400)		

PCP—Pentachlorophenol; NF—not found.

[a] Probably available but were not found in the selection of literature examined for the current review.

[b] Classification of the potential hazard according to the lowest median L(E)C50 values (all test organisms).

[c] Classification of the potential hazard according to the lowest median L(E)C50 values (crustaceans, bacteria and algae).

[d] Classification under Directive 67/548/EEC. The classification is carried out according to the lowest effect concentration. Substances are classified as dangerous for the environment and labeled with the symbol N (dangerous for the environment) and an adequate risk phrase (R). R50—very toxic to aquatic organisms; L(E)C50 < 0.1 mg/l; R53—may cause long-term adverse effects in the aquatic environment.

[e] Classification under Regulation No. 1272/2008 (European Parliament, 2008).

is actually a relatively low variation. Within the group of crustaceans (*D. magna, D. pulex, Ceriodaphnia dubia, Thamnocephalus platyurus, Chydorus sphaericus*, 10 values as a total; Table S1 available as the Supporting information) acute (24–48 h) LC50 values varied about 4 orders of magnitude for nano TiO_2 particles and about 3 orders of magnitude for C60 fullerenes (*D. magna, D. pulex, T. platyurus*, altogether 5 values; Table S1—Supporting information and Fig. 4C).

For the comparison, acute toxicity data of Cu^{2+} (48–96 h LC50) for fish (zebrafish, rainbow trout, trout, common carp, gibel carp, mullet, 13 values; Table S4—Supporting information) varied also almost two orders of magnitude. Toxicity of copper on fish varies due to the species differences but also as toxicity of dissolved copper is determined in large part by its chemical speciation. Even within the same species, the results vary substantially, depending upon the water chemistry, the testing protocol, and the life stage of the test organisms (Riedel, 2008). The high variability of (eco)toxicological data is also related to the variability of experimental conditions used. Indeed, experimental protocols, especially concerning preparation/suspending of NPs (addition and removal of solubilisation vehicles, filtering, centrifugation, sonication, dialysis) varies from paper to paper and has sometimes strong effect on result of the toxicity test. Thus, due to the above mentioned variability, this review does not aim to validate the collected data but rather provides quantitative information on ecotoxicological effects of NPs available in the papers associated with references for further information. Thus, the validation (if needed) is the responsibility of the user of this information.

6.3. Response of environmentally relevant test organisms: non-vertebrates and fish

As described above, regulatory testing of ecotoxicological hazard of pure chemicals involves testing with fish, *Daphnia* and algae (test species representing different food-web level). The toxicity data for these three groups were evaluated as well as those for bacteria—representatives of decomposers. As bacterial tests are probably the most cost-effective, individual toxicity data of NPs and particles of bulk ZnO, CuO and TiO_2 are presented in Fig. 4 in comparison to the bacterial median effect values. Individual values of toxicity of particles for bacteria were also plotted in the same manner as a reference and to indicate variability.

The coefficients of the linear regression log(EC50)–log(median EC50 bacteria) equations (C60 excluded) for the data presented in Fig. 4 are as follows:

	Bacteria	Algae	Crustaceans	Fish
Number of values	22	14	28	11
Slope	1.136 ± 0.177	1.032 ± 0.182	1.229 ± 0.206	0.782 ± 0.168
Y-intercept	-0.375 ± 0.367	-1.977 ± 0.418	-1.656 ± 0.530	-0.329 ± 0.425
r^2	0.674	0.728	0.579	0.706

This QAAR plot (Fig. 4) shows that good correlation exists for all these organisms and that algae were most sensitive organism group (see also Fig. 5).

Thus, although the NPs differ from bulk chemicals and show specific biological and environmental effects, the comparison of their toxicities (median values) shows some common tendencies: the most sensitive environmentally relevant species for NPs (Table 2) as well as for the reference chemicals (Table 3) were algae and crustaceans. The same tendency was observed by Hutchinson et al. (2003) for active pharmaceutical ingredients (APIs): for 73 of the 91 APIs, the algal median effect concentration (EC50) and daphnid EC50 values were lower than or equal to the fish LC50 data. Thus, for approximately 80% of these APIs, algal and daphnid acute EC50 data could have been used in the absence of fish LC50 data to derive PNEC (predicted-no-effect concentration) water values. Analogously, Jeram et al. (2005) evaluated the acute toxicity for fish, daphnids and algae data from New Chemicals Database of the European Chemicals Bureau. Analysis of the sub-set of data (496 compounds) with precise L(E)C50 values for both algae and daphnia test results available showed that 401 out of the 496 substances acute algal EC50 and daphnid EC50 values were lower than or equal to the fish LC50 data meaning that in only in 91 (18.3%) cases fish was the most sensitive species. Hoekzema et al. (2006) also evaluated toxicity data sets for 507 compounds, including agrochemicals, industrial chemicals, and pharmaceuticals from their internal database and showed that in 188 (90%) of the 208 cases for which a complete data set was available, the median effect concentration for algae or daphnids was lower than the LC50 for fish. Therefore, Hutchinson et al. (2003) suggested that the current regulatory requirement for fish LC50 data regarding APIs should be succeeded by fish acute threshold (step-down) test data, thereby achieving significant animal welfare benefits with no loss of data for PNEC estimates.

The above described research has recently yielded modification of the OECD guideline 203 on acute toxicity testing of chemicals using fish (OECD, 2008). According to this modified guideline, the fish test would be performed only at one concentration, the lowest

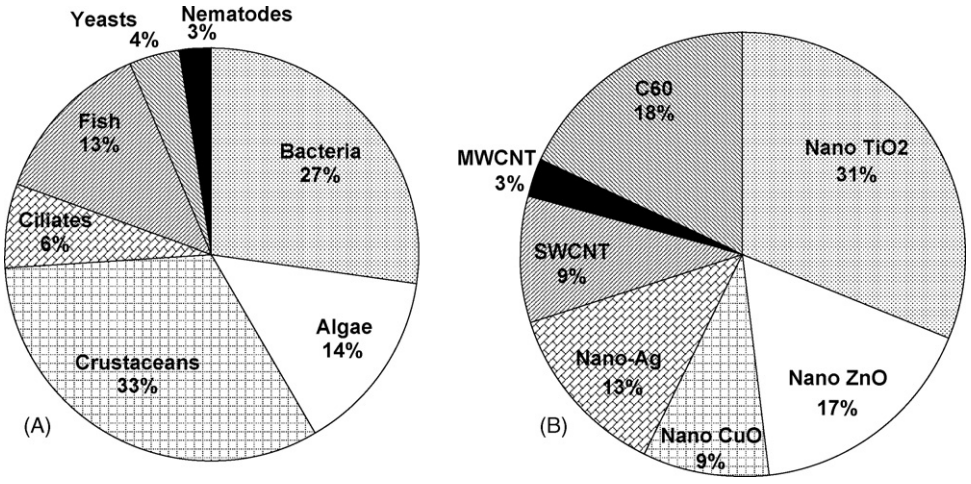

Fig. 3. (A and B) Distribution of quantitative ecotoxicological data for synthetic nanoparticles between organism groups (A) and particle-wise (B). Total number of records was 77. Data are plotted from Table 2.

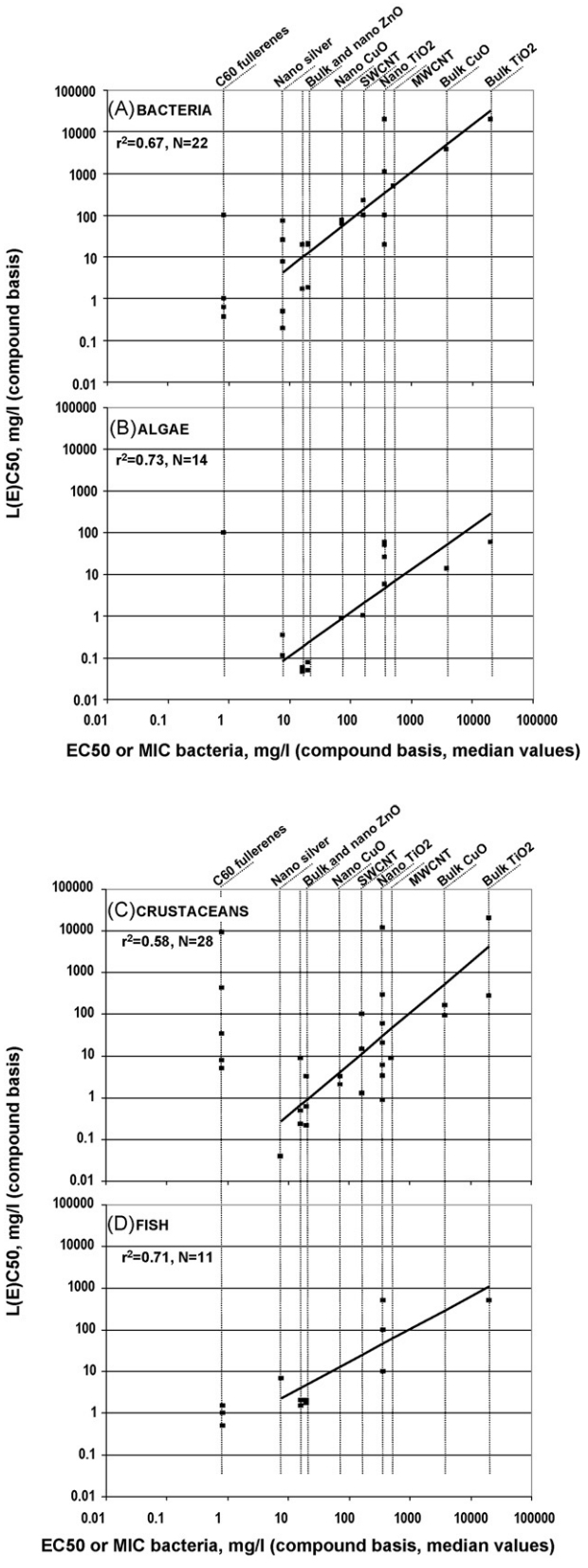

Fig. 4. Individual L(E)C50 values of various (nano)particles towards different ecotoxicological test organisms (bacteria, algae, crustaceans, fish) *versus* the median L(E)C50 values for bacteria. Data are plotted from Tables S1–S4. Dotted vertical lines point to the corresponding particle.

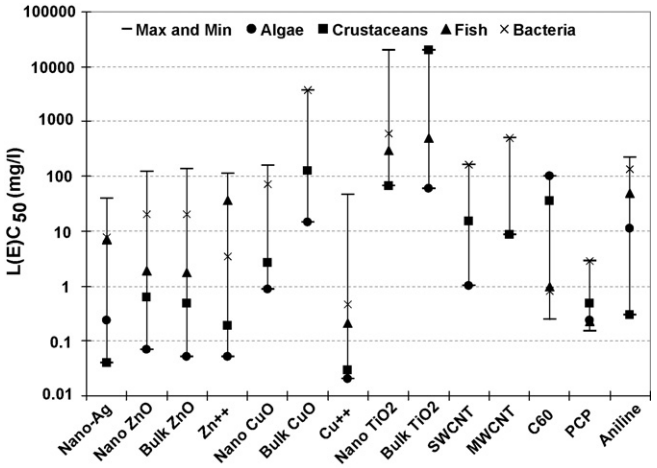

Fig. 5. Median and variation of L(E)C50 values of nanoparticles and reference compounds to different organism groups. Data are plotted from Tables 2 and 3.

between the EC50 concentrations obtained with previous testing with algae and daphnia. When fish would be more sensitive than algae and daphnia, testing with fish would be continued at lower concentrations (step-down). Currently, revised OECD guideline 203: fish, acute toxicity test involving the threshold approach, that takes into consideration EC50 values from relevant algae and acute invertebrate (*e.g.*, daphnia) tests, is in the review phase. As algae and daphnids were most sensitive species for synthetic NPs (except for C60 fullerenes for which ciliates were most sensitive; Table 2), the step-down approach of OECD 203 seems to be reasonable also for evaluating the toxicity of NPs to fish. However, as the number of available data for fish is currently quite limited, more research is needed.

The most toxic NP for algae and nematodes was nano ZnO. For bacteria, fish and ciliates the most toxic was C60 fullerene and for the crustaceans, nano Ag (Table 2). However, plotting the range of L(E)C50 median values for different groups of organisms and all the studied compounds (nano and not nano; Tables 2 and 3; Fig. 5) shows that there is no big difference between the toxicities of NPs and their respective bulk formulations except in the case of CuO. The most toxic particles were nano Ag, ZnO, C60-fullerenes and nano CuO (Fig. 5). Both TiO_2 (nano and bulk) were less toxic but still were classified as harmful (see below). The few available data indicate that carbon nanotubes are probably less toxic than fullerenes and metal-containing NPs. One should observe the very high toxicity of NPs (at least to some organisms) comparable to well known dangerous biocides pentachlorophenol or copper (Fig. 5; Tables 2 and 3). As NPs show harmful effects at very low concentrations, they can lead to disruption of the food-chain leading further to global effects concerning the entire ecosystem. From all the compounds studied, the most toxic was Cu^{2+} and the toxicity of copper ions varied from 0.02 mg/l (algae) till 45 mg/l (nematodes; Table 3). This is quite coherent with the difference in toxicity of copper ions to several freshwater and saltwater tests organisms reported by Riedel (2008): from 0.005 to 10 mg/l.

6.4. Ranking of the NPs into different toxicity categories

In EU, the primary objective of classifying substances and preparations dangerous to the environment is to alert the user to the hazards these substances and preparations present to ecosystems. Directive 93/67/EEC laid down the principles for assessment of risks to man and the environment of substances notified in accordance

with Council Directive 67/548/EEC (CEC, 1996). The new regulation 1272/2008 (European Parliament, 2008) amended and replaced the Directive 67/548/EEC for harmonising the classification, labeling and packaging of substances and mixtures with the UN Globally Harmonised System (GHS).

As mentioned briefly above, the ecotoxicological classification is based on toxicity to fish, *Daphnia*, and algae, as well as data about biotic and abiotic degradability of the substance. Under Directive 67/548/EEC a substance was classified as "harmful", "toxic" or "very toxic" to aquatic organisms depending on the 96-h LC50 for fish, 48-h EC50 for daphnids, and 72-h EC50 for algae. As a result, substances showing certain biological effects were classified as dangerous for the environment and labeled with the symbol N (dangerous for the environment) and an adequate risk phrase (R). If L(E)C50 values were <1 mg/l, a substance was classified as "very toxic to aquatic organisms" (danger symbol N, risk phrase R50). If the values obtained for toxicity were between 1 and 10 mg/l, a substance was considered "toxic to aquatic organisms" (danger symbol N, risk phrase R51) and if the L(E)C50 values were between 10 and 100 mg/l, a substance was classified as "harmful to aquatic organisms" (risk phrase R52). This classification was carried out according to the lowest effect concentration. In the new regulation 1272/2008 the limits of toxicity remain similar but the wording of the classification is different (see Table 3).

In the scientific research this standard approach has also been used and shown that at least for some chemicals (arsenic and 1,4-butynediol) additional data about environmentally relevant properties could lead to a revision of present chemical classification and labeling (Tišler and Zagorc-Končan, 2003). The above described standard approach has been also modified, by adding more tests as well as adding additional toxicity categories. For example, the same toxicity grid but different test battery has been used for ranking of eight phenolic compounds by Kahru et al. (2000) and the same toxicity grid was applied for the comparison of toxicity of five inorganic and organic NPs to bacteria *V. fischeri* by Mortimer et al. (2008). Using toxicity data for fish, daphnids and algae but applying slightly different grid Sanderson et al. (2003) have classified pharmaceutical compounds and using various ecotoxicological test organisms Blaise et al. (2008) classified eleven nanomaterials using the following grid: L(E)C50 < 0.1 mg/l = extremely toxic to aquatic organisms; 0.1–1 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 = non-toxic to aquatic organisms.

In this review, the grid applied by Sanderson et al. (2003) and Blaise et al. (2008) was applied for the potential ecotoxicological hazard evaluation of seven NPs (Table 2): the most harmful were nano Ag and nano ZnO that were classified "extremely toxic", (L(E)C50 < 0.1 mg/l), followed by C60 fullerenes and nano CuO that were classified "very toxic", (L(E)C50 0.1–1 mg/l). SWCNTs and MWCNTs were classified "toxic" (L(E)C50 1–10 mg/l). Nano TiO_2 was classified "harmful", (L(E)C50 10–100 mg/l). For the comparison, also reference compounds (bulk metal oxides, Zn^{2+}, Cu^{2+}, aniline and pentachlorophenol) were classified using analogous approach that was applied for NPs and compared with the previous official risk phrases and current official hazard classes for these compounds (if available in EU respective documents). In general, estimates were in good agreement (Table 3). Interestingly, analysis of the data presented in this review (Tables 2 and 3) shows that a reduced test battery involving just three types of organisms (algae, bacteria, crustaceans—representatives of three different food-web levels) could be enough predictive as a full suite of 7 organism groups, to yield these hazard rankings for these NPs but also for the reference chemicals.

6.5. Response of ecotoxicologically relevant test organisms: effect of solubility of some (nano)particles

For NPs of ZnO, TiO_2 and CuO, algae and crustaceans were the most sensitive test organisms (Table 2 and Fig. 6). Algae were also most sensitive organisms for bulk ZnO, bulk CuO, bulk TiO_2, Zn^{2+} and Cu^{2+} (Table 3). Moreover, there is a close relationship between toxicity of nano metal oxide and the corresponding bulk formulation. The coefficients for the linear regressions log(nano)–log(bulk) (data in Fig. 6) are as follows:

	Number of values	Slope	Intercept Y	r^2
ZnO	13	0.865 ± 0.110	0.0289 ± 0.114	0.849
CuO	7	0.900 ± 0.134	−1.291 ± 0.379	0.900

In addition, whatever was the group of test organisms the toxicity of both, bulk and nano ZnO practically did not differ and was most probably due to the solubilisation of Zn-ions from the respective oxides that was also stated previously by several authors (see below). However, the nano CuO was as median, 51-fold more toxic than bulk CuO (16-fold difference for algae and 48-fold difference for crustaceans) whereas there was no difference between particle-ingesting and not ingesting organisms (Table 2).

Using recombinant sensor bacteria for quantification of bioavailable ions, it has been previously shown that, as a rule, the toxicity of these metal-oxide NPs and of their respective bulk formulations to crustaceans *D. magna* and *T. platyurus*, bacteria *V. fischeri* (Heinlaan et al., 2008; Mortimer et al., 2008), algae *P. subcapitata* (Aruoja et al., 2009), protozoa *T. thermophila* (Mortimer et al., 2010) as well as to yeast *S. cerevisiae* (Kasemets et al., 2009) was mainly explained by solubilisation of zinc and copper from these particles. Analogous effect for nano and bulk ZnO was shown by Wang et al. (2008) for nematodes *C. elegans*. Thus, at least for ZnO and CuO NPs (but probably also for other metal oxides) it seems reasonable to derive the initial toxicity threshold data from the solubilities of these NPs in different test/exposure media. As there is a number of ecotoxicity data for Zn and Cu available (Table 3) and a lot of research already made on effects of chemical speciation on

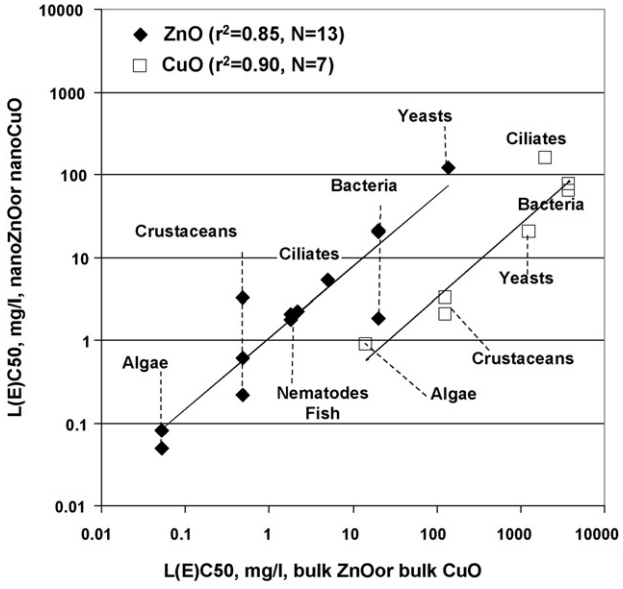

Fig. 6. Individual L(E)C50 values of nano CuO and nano ZnO *versus* bulk CuO and bulk ZnO to different organism groups. Data are plotted from Tables S1–S6. Dotted lines point to the corresponding organism group.

bioavailability of these metals, these data may be all used, at least as an initial step. Interestingly, concerning the comparison of the effects of nano and bulk CuO, Fig. 6 points out an outlier—the ciliate *T. thermophila*. Indeed, protozoa *T. thermophila* (particle-feeding organisms) were intensively moving even when their food vacuoles were full of ingested aggregates of nano CuO. It could be assumed that nanosized CuO particles were more readily ingested by *T. thermophila* than bulk CuO particles and thereby more rapidly removed from the medium (Mortimer et al., 2010). Thus, for these organisms the internalization seems to sequester the harmful NPs and thus lower toxicity. Similar observation was reported already long time before the nanotoxicology era by Nilsson (1978): the high tolerance of *Tetrahymena* towards lead (Pb) was believed to be due in part to the low ionic concentration of lead under the test conditions and in part to a "detoxication mechanism" consisting of retention of lead within the digestive vacuoles.

7. Concluding remarks

The use of NPs is constantly increasing in broad applications. As for bulk chemicals, the life cycle of NPs/nanomaterials will involve various environmental compartments. Therefore, (eco)toxicological information is required at several levels (single organisms, simplified communities and whole ecosystems) for risk assessment and regulatory purposes. This review summarized currently available quantitative ecotoxicological data. In addition, the review pointed out the existing strategic information accumulated during several decades of ecotoxicological studies on bulk chemicals (choice of test organisms, validation of test protocols) as well as by introducing recent toxicogenomic methods, to obtain new mechanistic knowledge on NP-induced stress response in relevant model organisms. All that information should be taken on board.

Quantitative data on toxicological effects of NPs are still scarce even at the single organism level. The most sensitive test organisms towards NPs were algae and crustaceans revealing the vulnerability of these organism groups in the aquatic food-chain. The latter was true not only for the 7 different types of NPs but also for the 7 reference chemicals. Thus, the step-down approach of Hutchinson et al. (2003) could also be adaptable for evaluation of ecotoxicological effects of NPs to fish.

The currently existing quantitative nanoecotoxicological data on single model organisms would classify NPs from "extremely toxic" to "harmful". Remarkably, none of the NPs studied in this review was classified "not harmful". In such a classification some of these NPs proved as toxic or even more toxic than well known dangerous biocide pentachlorophenol (PCP) that has already been banned or severely restricted for health and/or environmental reasons in most countries (UNEP, 1996). Although some synthetic NPs are already widely used in various consumer products/applications, their real environmental impact remains almost unexplored. Laboratory results show that even aggregated, NPs can be toxic due to their solubilisation or other specific properties. But one black box remains to be opened and understood: the environmental fate of NPs which is modulating their environmental impact. Currently, neither the fate of nanosize materials nor their impact on animals, plants and soil communities have been investigated *in situ* although it would be necessary for the validation of models proposed for the environmental risk assessment of NPs.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.08.016.

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