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Mapping the Dawn of Nanoecotoxicological Research

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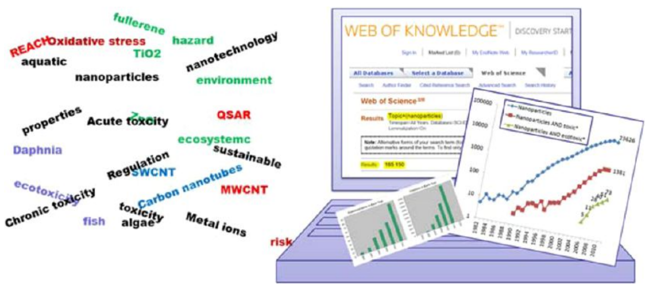

Some researchers consider nanotechnology the next industrial revolution, and consumer products and a variety of industries increasingly use synthetic nanoparticles. In this Account, we review the initial accomplishments of nanoecotoxicology, a discipline that is just a decade old. This new subdiscipline of ecotoxicology faces two important and challenging problems: the analysis of the safety of nanotechnologies in the natural environment and the promotion of sustainable development while mitigating the potential pitfalls of innovative nanotechnologies. In this Account, we provide a snapshot of the publicly available scientific information regarding the ecotoxicity of engineered nanoparticles. We pay special attention to information relevant to aquatic freshwater species commonly used for risk assessment and regulation.

Just as the development of ecotoxicology has lagged behind that of toxicology, nanoecotoxicological research has developed much more slowly than nanotoxicology. Although the first nanotoxicolology papers were published in 1990s, the first nanoecotoxicology papers came out in 2006. A meta-analysis of scientific publications covering different environmental impacts of nanomaterials showed that the importance of research into the environmental impact of nanotechnology has gradually increased since 2005. Now the most frequently cited papers in the environmental disciplines are often those that focus on synthetic nanoparticles.

The first nanoecotoxicology studies focused on adverse effects of nanoparticles on fish, algae and daphnids, which are ecotoxicological model organisms for classification and labeling of chemicals (these model organisms are also used in the EU chemical safety policy adopted in 2007: Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH)). Based on our experience, we propose a multitrophic battery of nanoecotoxicological testing that includes particle-feeding and a priori particle-"proof" prokaryotic and eukaryotic organisms at different food-chain levels. Using this battery of selected test organisms, we demonstrated that TiO_2 nanoparticles were toxic to algae and that ZnO and CuO nanoparticles were toxic to several aquatic invertebrate test species. Thus, one single biotest cannot predict the ecotoxicological effects of chemicals/nanoparticles, and researchers should use several tests instead. Moreover, produced nanoparticles usually vary in features such as size, shape, and coating; therefore, a single nanoparticle species may actually include many entities with different physicochemical properties. An ecotoxicity analysis of all these variants would require a huge number of laboratory tests. To address these issues, high throughput bioassays and computational (QSAR) models that serve as powerful alternatives to conventional (eco)toxicity testing must be implemented to handle both the diversity of nanomaterials and the complexity of ecosystems.

1. Introduction

The current Account aims to provide the reader with the following:

(i) A snapshot on the current scientific knowledge regarding the ecotoxicity of engineered nanomaterials (NMs) with a focus on aquatic freshwater species

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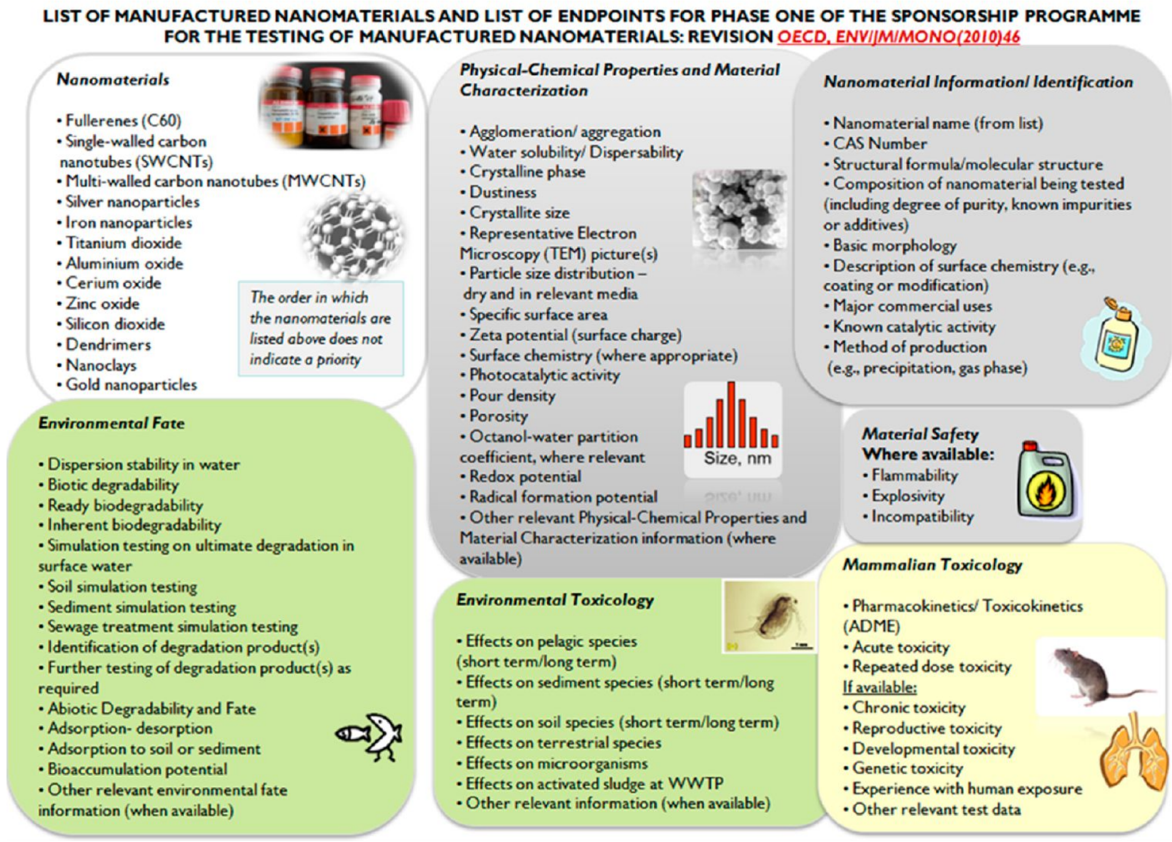


FIGURE 1. Schematic summary of the main information from the revised version of the List of Manufactured Nanomaterials and List of Endpoints for Phase One of the Sponsorship Programme for the Testing of Manufactured Nanomaterials,[4] originally published in 2008 and updated in 2010.

commonly used for risk assessment and for regulation. Other important areas of ecotoxicology, such as terrestrial toxicity, bioaccumulation, biodegradation, as well as fate and transport of NMs in the environment (Figure 1), are marginally addressed in this Account, mostly due to the shortage of respective information for synthetic NMs in the literature. Moreover, the topic of the biodegradability of NMs only encompasses that for carbon-based NMs and/or organic coatings of metallic NMs.

(ii) A short literature-based mapping of the onset and evolution of nanoecotoxicological research during the past decade, within the scope of environmental disciplines.

The findings are illustrated by two case studies which addressed the (eco)toxicological hazard of synthetic nanoparticles (NPs): bibliometric and experimental examples. Special attention in these studies is drawn on the adverse effects on invertebrate aquatic species (daphnids, algae, bacteria, protozoa) by, for example, TiO_2, ZnO, CuO, and nanosilver that are already incorporated into a variety of consumer products (ref 1 and references therein). The selected aquatic species are significant both for the ecological food chain and as model organisms in regulatory testing.[2,3]

The term "ecotoxicology" was coined by René Truhaut in 1969.[5] Ecotoxicology is a relatively new science that deals with the effects of toxic chemicals on organisms other than man, especially at the population, community, and ecosystem levels. Ecotoxicology has become an important component of environmental risk assessment via provision of data on the direct effects of toxicants on a base set of model test species. This is important as managers and risk assessors need tools and information that can be applied readily to a decision-making process in a timely manner. However, there has been a clear reluctance among ecotoxicologists to address the complexity of ecosystem through experimentation and use of model species.[6] Indeed, from the perspective of all ecosystems, one has to consider that while risk assessment for humans addresses only a single species, environmental risk assessment concerns millions of species, with different morphology, physiology, nutritional habits, stress-susceptibility, and ecological habitats.

At 2003, Van Straalen wrote: "Because the major environmental pollutants, at least in Europe and North America, are coming under the control of regulatory authorities and are declining, this part of ecotoxicology (i.e., the testing-based-approach) is now more or less completed."[7] But then society faced new "emerging toxicants", and by 2007 more than 60 countries had started national nanotechnology programs. Currently, worldwide annual total public and private sector funding for nanotechnologies is about \$13–14 billion (http://www.nanowerk.com/nanotechnology/ten_things_you_should_know_6.php). Indeed, in the midst of a global economic recession, exponential population growth, widespread food, feed, fuel, and raw materials shortages, environmental deterioration, and societal problems, nanotechnologies are expected to make an impact in each of these domains and have been referred to as the next industrial revolution.[8] According to the Nanotechnology Consumer Products Inventory (http://www.nanotechproject.org/consumerproducts), there are currently 1317 "nano"-products produced by 587 companies located in 30 countries. The contribution of nanotechnology to the global economy is expected to grow to \$3.1 trillion by 2015.[9]

The great expectations for nanotechnology are based on the fact that at the nanoscale materials assume novel and enhanced properties compared to the bulk material. This is due to an increased relative surface area and surface display of their ingredient atoms, which translates into higher surface reactivity and display of new electronic, optical, quantum mechanical, and magnetic properties. However, the physicochemical properties that are responsible for technological breakthroughs could also lead to increased bioavailability and toxicity of engineered NPs.[10] Therefore, dealing with uncertain risks from engineered NMs to the environment is an important, critical and challenging task.

The necessity to perform a rigorous analysis of benefits and risks in order to guarantee the sustainability of nanotechnologies was understood already in 2004, when a large insurance company Swiss Re released a report "Nanotechnology—Small matter, many unknowns" and in the same year The Royal Society and Royal Academy of Engineering published a report "Nanoscience and nanotechnologies: opportunities and uncertainties".[11] Recently (2011), European Academies Science Advisory Council and EC Joint Research Centre in their report "Impact of engineered nanomaterials on health: considerations for benefit-risk assessment" described the state-of-the art knowledge on the safety aspects of engineered NMs.[12] They reported big knowledge gaps in the safety aspects of engineered NMs and the need for further scientific investigations. Indeed, the lack of adequate safety information may ultimately counteract any preliminary gains and hamper the sustainable development of nanotechnologies. The development of toxicological as well as ecotoxicological research in EU has been facilitated by the new EU chemical safety policy, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) introduced in 2007.[13] According to REACH, by 2018, all chemical substances manufactured or imported in quantities of more than 1 metric ton per year in the European market must be characterized for their potential impact on aquatic ecosystems. This includes requirements for short-term toxicity data for crustaceans (preferred species *Daphnia*) and growth inhibition data for aquatic plants (algae preferred). In addition, short-term toxicity testing on fish is required for the next annual tonnage level (>10 metric tons). The number of chemicals that require ecotoxicological assessment by 2018 is a matter of debate with estimations ranging from 30 000 to more than 100 000 substances.[14] Although there are no provisions in REACH referring specifically to nanomaterials, it deals with substances, in all sizes, shapes, or physical states. Thus, it follows that under REACH and the new Regulation 1272/2008 manufacturers, importers, and downstream users have to ensure that their NMs do not adversely affect human health and/or the environment. As the current priorities are directed toward the analysis of benefits and risks of NMs, regulatory tests have become prevailing in nanoecotoxicology. However, mechanistic studies, such as, ecotoxicogenomics, are emerging to elucidate the impact of NMs on biological receptors.[15]

The OECD, that has a wealth of experience in developing methods for the safety testing and assessment of chemicals, established in 2006 The Working Party on Manufactured Nanomaterials (WPMN) to help member countries efficiently address the safety challenges of NMs. In 2007, the WPMN launched the Sponsorship Programme for Testing on Manufactured Nanoparticles and agreed on a priority list of NMs and a list of end points relevant for human health and environmental safety that should be tested.[16] The WPMN panel of "Environmental Toxicology" comprises the effects on pelagic, sediment, soil, and terrestrial species, microorganisms, and activated sludge (Figure 1).

2. Information on Nanosafety to Humans and The Environment: A Bibliometric Approach

As of the end of 2011, Thomson Reuters ISI Web of Science (ISI WoS) listed the total number of papers that could be retrieved through the use of the search term "nanoparticles" as ∼160 000. This represented a doubling of the number of such publications since March 2009, at which point 83 295

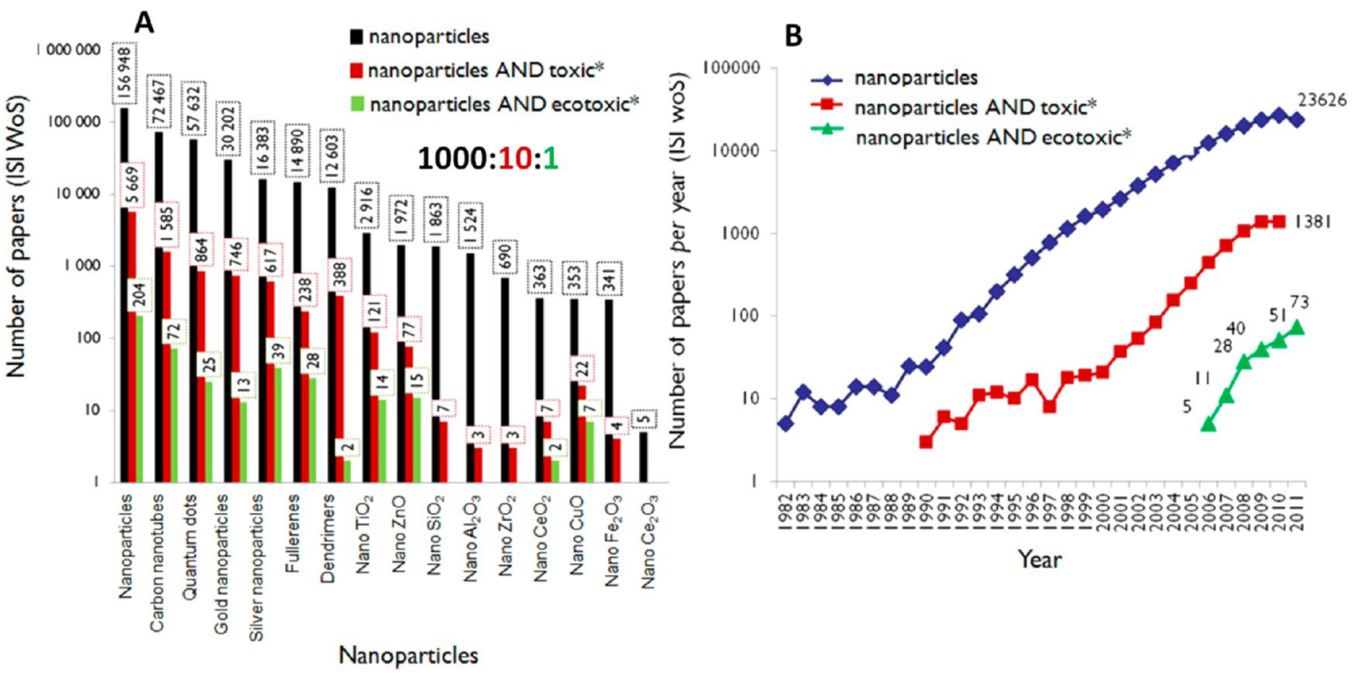


FIGURE 2. (A) Information registered in ISI WoS on various types of nanomaterials since 1980. The search for each type of nanomaterial (black bars) was refined (i) using term "toxic*" to retrieve the respective toxicological information (red bars) and (ii) "ecotoxic*", to obtain respective ecotoxicological information (green bars). Search was performed on October 16, 2011. The values on the bars refer to the number of documents found. Approximate ratio of papers on "nanoparticles", "nanoparticles"AND "toxic*" and "nanoparticles" AND "ecotoxic*" is shown. (B) Timeline of the emergence of scientific information on nanotoxicological and nano*eco*toxicological aspects of nanoparticles: number of publications (per year) in Thomson Reuters ISI WoS on search terms indicated. Search was performed on October 23, 2011.

documents were retrieved under the same terminology.[3] Thus, the doubling time for the expansion of nanotechnological information may be as short as 2.5 years. Figure 2A shows that currently the most extensively studied NPs are carbon nanotubes (CNTs), quantum dots, gold and silver NPs, fullerenes, and dendrimers. Within metal oxide NPs, most scientific research has been performed on TiO_2, followed by ZnO, SiO_2, and Al_2O_3. All these NPs are also on the OECD "priority" list (Figure 1). The prevalence of papers on CNTs and silver NPs is consistent with their extensive use in consumer products, whereas the large amount of information on quantum dots, fullerenes, dendrimers, and gold NPs can be explained by their potential application toward medicine (e.g., bioimaging, targeted cellular drug trafficing and cancer medication).[10] The safety aspects of manufactured NMs are important from both human and environmental perspectives. Humans as producers and consumers of nanoenabled products are the first to be in contact with synthetic NMs, whereas exposure of other biological species could occur when the NMs are released, for example, by various industrial and household waste-streams. Despite the importance of NM safety aspects, the current information on potential harmful effects of NMs is relatively scarce. From 160 216 papers that were available in ISI WoS for search term "nanoparticles" in the time frame from 1980 until November 2011 (Table 1), about 1/3 of them concerned nanoparticles' properties (53 619 records) or structure (39 472), that is, were related to the research in the materials aspect of NPs. Only 5832 (3.6% of papers on "nanoparticles") addressed the search term "toxic*", 0.84% of papers were related to "health", 0.64% to "safety", and 0.18% to "hazard". Among the most cited papers related to NM safety was the pioneering review "Toxic potential of materials at the nanoscale level"[10] which has been designated as a current classic by Thomson Reuters on two occasions. The other two most cited papers on nanosafety[17,18] concerned the medical aspects of nanoresearch (Table 1).

Compared to the existing nanotoxicological information, information on the ecotoxicological aspects of NPs is even more scarce. When we studied the share of respective information among all the scientific papers published for each type of nanomaterial (additional search terms "toxic*" and "ecotoxic*" were used), we noted that the average share of nanotoxicological papers was 1% and nano*eco*toxicological ones about 0.1% (Figure 2A). Thus, as a rule of thumb, irrespective of the type of the nanomaterial, for every 1000 NP-papers about 10 included information on their toxicological effects and 1 on ecotoxicological properties.

TABLE 1. Number and Citation Analysis of Papers Concerning Different Aspects of Synthetic Nanoparticles[a]

search term[b]	papers no.	papers %	the most cited paper[c] authors (citations[c])	the most cited paper[c] research focus
nanoparticles	160 216	100		[d]
papers on search term "nanoparticles" refined by following search terms:				
properties	53 619	33		[d]
structure	39 472	25		[d]
toxic*	5832	3.6	Nel et al.[10] (1235)	nanotoxicity
health	1339	0.84	Moghimi et al.[17] (422)	nanomedicine
safety	1025	0.64	Lee et al.[18] (372)	biomedicine, collagen
hazard	282	0.18	Lam et al.[19] (284)	carbon nanotubes, occupational and environmental health risks
sustainab*	277	0.17	Balazs et al.[20] (452)	nanocomposites
ecotoxic*	211	0.13	Nowack and Bucheli[21] (243)	environmental fate
occupational	188	0.12	Lam et al.[19] (284)	see row above
ecosystem	109	0.07	Blaser et al.[22] (94)	silver, plastics, textiles, risks
epidemiolog*	93	0.06	Stoeger et al.[23] (135)	carbon nanoparticles, pulmonary toxicity
life cycle assessment	42	0.03	Müller and Nowack[24] (175)	exposure modeling, environment
other	57 727	36		not applicable

[a]Search in Thomson Reuters ISI WoS was performed in November 22, 2011. [b]On field "topic". [c]as by November 22, 2011. [d]Citation analysis was not possible (>10 000 documents). (*) was used to truncate search terms.

In total, the bibliometric search using terms "nanoparticles" and "ecotoxic*" yielded only 211 papers (Table 1). The first nanotoxicolological papers were published in 1990s, whereas the first nano*eco*toxicologial papers emerged in 2006 (Figure 2B). Thus, similar to the development of ecotoxicology, which has been severely lagging behind toxicology,[3] nanoecotoxicological research lags behind nanotoxicology by at least 10 years.

3. Nanoecotoxicology: an Emerging Discipline

Nanoecotoxicology is a very recent discipline that studies the ecotoxicity of nanomaterials and can fortuitously rely on the solid research data on ecotoxic effects of "regular" chemicals collected over decades by environmental chemists, ecologists, biologists, as well as material scientists and physicists. That statement is especially relevant for metallic NPs as metals have been extensively studied for their biological effects.[3]

To analyze the evolution of environment-related "nano"-research in more detail, a bibliometric analysis of the papers published in selected six environmental journals: *Environmental Science and Technology* (*ES&T*), *Chemosphere*, *Environmental Pollution*, *Science of the Total Environment*, *Aquatic Toxicology*, and *Water Research* between 2002 and October 2011 was performed (Figure 3A). More detailed study on the share of "nano"-papers was conducted for two journals: *ES&T* and *Chemosphere*. The total number of papers published within this time frame in these six journals was 12 759 (data not shown). While in 2002−2003 the share of "nano"-papers was 1−2%, in 2009−2011 already 5−6% of papers in *Chemosphere* and 10−12% of papers in *ES&T* concerned nanomaterials (Figure 3B). To follow the timeline of advent of environment-related "nano"-research and its main research foci since 2002, the research topics/keywords of the most cited papers in the above highlighted six journals (as by October 2011) were mapped for each year (Figure 3A). The most important topics covered over the past 9 years were related to (i) antibiotics, pharmaceuticals, and endocrine disrupters (14 highly cited papers, accumulating 4492 citations), (ii) other toxic pollutants such as arsenic, mercury, and phenols (13 papers with 2662 citations), (iii) synthetic nanomaterials (12 papers with 1599 citations), (iv) brominated flame retardants (4 papers with 2174 citations), followed by microbial fuel cells and atmospheric pollution (4 papers with 867 citations). According to the citation pattern, the priority of the environment-related research switched to "nano" by 2005, when most cited papers in *ES&T* and *Aquatic Toxicology* concerned synthetic NMs. Remarkably, since 2006 at least one "nano" paper was included annually among the most cited papers in one of these six journals. Analysis of the types of NMs and research topics of the highly cited "nano"-papers (Figure 3A) allowed us to establish a timeline for the development of research priorities in environmental "nano"-science.

The first highly cited "nano"-papers concerned cytotoxicity analysis of single- and multiwall carbon nanotubes (SWCNTs and MWCNTs) and impact of fullerenes on alveolar macrophages;[25] toxic effects of SWCNTs and TiO_2 NPs to rainbow trout;[26,27] ecotoxicity of TiO_2, SiO_2, and ZnO NPs to bacteria;[28] effects of coexposure of TiO_2 NPs and fullerenes to *Daphnia magna*;[29] stabilization of CNTs by natural organic

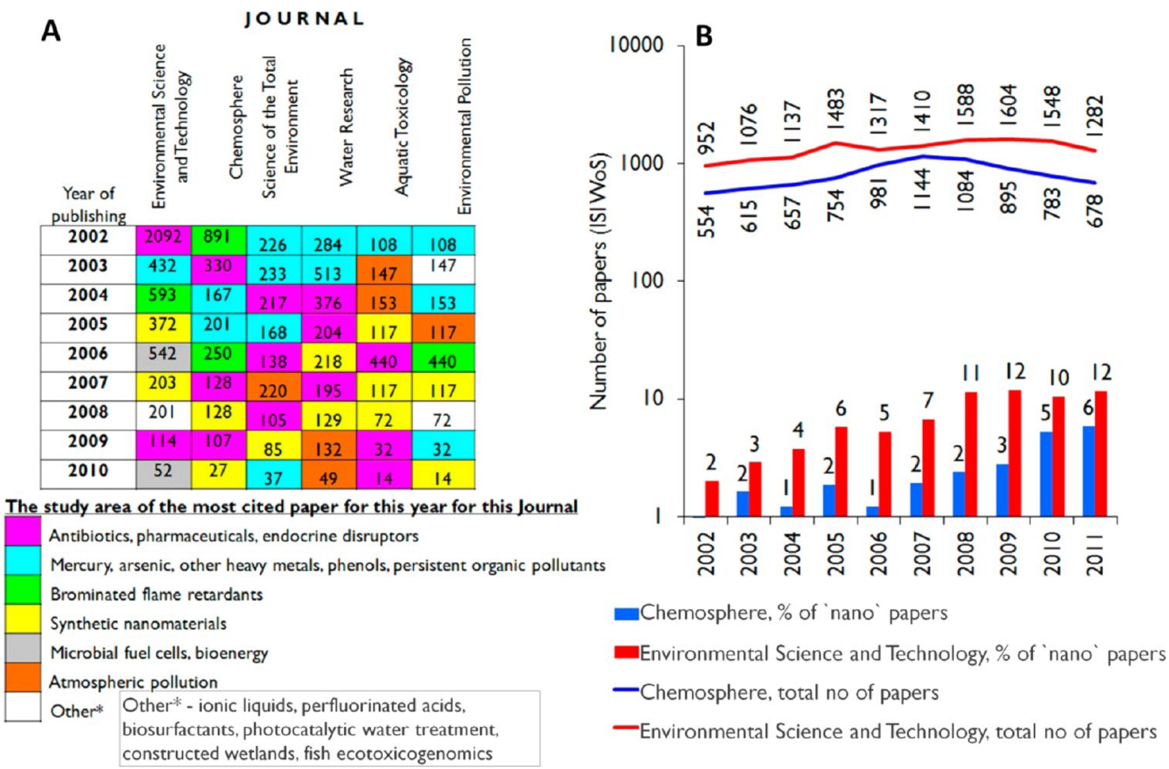
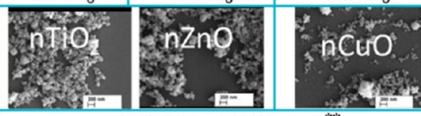


FIGURE 3. Evolution of the "nano"-topics of the environment-related research since 2002. (A) Focus of the research of the most cited paper for the given year in six environmental journals: *Environmental Science and Technology*, *Chemosphere*, *Science of the Total Environment*, *Water Research*, *Aquatic Toxicology*, and *Environmental Pollution*. On the respective field also the cumulative number of citations for the given paper as by October 24, 2011 is presented. (B) Bibliometrical study of the timeline for onset and development of "nano"-research since 2002. Number of papers published annually and percentage of "nano"-papers in *Environmental Science and Technology* and *Chemosphere*.

matter;[30] behavior, fate, and environmental effects of nanomaterials;[21] toxicity of ZnO, CuO, and TiO_2 NPs to aquatic crustaceans and bacteria;[31] development of antimicrobial NMs for water disinfection/microbial control;[32] toxicity and bioaccumulation of organic xenobiotics in the presence of fullerenes;[2] toxicity of TiO_2, CuO, and ZnO NPs to algae;[33] ecotoxicity of CuO and ZnO NPs in natural water;[34] and toxicity and bioaccumulation of TiO_2 NPs in *D. magna.*[35] Here, it is interesting to compare the similarities in the development of ecotoxicology and nanoecotoxicology. The first study wave in both disciplines focused organism-wise on fish, algae, and daphnids which are ecotoxicological model organisms used for classification and labeling of chemicals, for example, currently also for the purposes of REACH. The same tendency toward "preference" of this base set of aquatic organisms was observed in the Report of ENHRES[36] that analyzed the respective newly emerged scientific literature published until December 2008, and similar recommendations were given by Handy et al.[37] At the same time, studies on other organisms, especially soil- and sediment-relevant organisms, lagged behind.[3] By October 2011, 35%, 29%, and 24% of the existing nanoecotoxicolological data concerned fish, algae, and daphnids, respectively. There were only 22 papers on protozoa, 32 on nematodes, 25 on earthworms, and no papers on springtails (*Collembolae*) (data not shown).

4. Multitrophic (Eco)toxicological Test Batteries in Nanosafety Research

4.1. An Experimental Case-Study on ZnO, CuO, and TiO_2 Nanoparticles. As mentioned above, synthetic NMs may end up in the environment, and there are vast data gaps concerning the adverse effects of NMs, especially to the environmentally relevant test organisms. To address these data gaps, the authors' research group has begun evaluating the hazard of, for example, nanoparticulate ZnO, CuO, and TiO_2 in various standard ecotoxicity organisms such as algae, daphnids, and bacteria, but also in less frequently used model organisms, such as yeast and protozoa (Figure 4). This set of test organisms represents (i) particle ingesting (daphnids, protozoa) and a priori particle-"proof' (bacteria, yeast, algae); (ii) organisms from different food-chain levels; (iii) pro- and eukaryotic organisms; (iv) regulatory species for ranking of environmental hazard of chemicals (algae, daphnids); (v) organisms widely used for QSAR-models



	NANOPARTICLES:	nTiO_2*	nZnO	nCuO
	CAS nr, appearance when purchased:	CAS 13463-67-7 White powder	CAS 1314-13-2 White powder	CAS 1317-38-0 Black powder
	Manufacturer, primary size (according to manufacturer):	Sigma-Aldrich, 25-70 nm	Sigma-Aldrich, 50-70 nm	Sigma-Aldrich, 30 nm
	Specific surface area (SSA) (BET):	24.8 m^2/g	12.9 m^2/g	25.5 m^2/g
Test, organism, toxicity endpoint and use of the test for regulatory purposes	**Potential for ecotoxicogenomics, Quantitative Structure Activity Relationship (QSAR) analysis and modeling**	**TOXICITY, L(E)C50, mg/L****		
Growth inhibition of algae *Pseudokirchneriella subcapitata* (OECD 201). A short-chronic test	Important for QSARs and regulatory purposes. Numerous toxicity data available	72 h EC50=9.73	72 h EC50=0.052	72 h EC50=0.89
Immobilization of crustaceans *Daphnia magna* (OECD 202). An acute test	Important for QSARS and regulatory purposes. Numerous toxicity data available. Commercially available as a ToxKit	48h EC50 ~20 000	48h LC50=3.2	48h LC50=3.2
Mortality of crustaceans *Thamnocephalus platyurus*. An acute test	Alternative test organism to *Daphnia magna*. Commercially available as a ToxKit	24h EC50 >20 000	24h LC50=0.18	24h LC50=2.1**
Decrease of viability (ATP content) of protozoa *Tetrahymena thermophila*. A short-chronic test	Macronucleus sequenced. Numerous toxicity data and QSARS available	24h EC50>1000	24h EC50=10.3	24h EC50=126
Growth inhibition of yeasts *Saccharomyces cerevisiae*. A short-chronic test	Popular *in vitro* toxicity model. Genome sequenced. Mutated strains (e.g., EUROSCARF) available	8h EC50>20000	8h EC50=121	8h EC50=20.7
Inhibition of bioluminescence of bacteria *Vibrio fischeri* NRRL B-11177. (ISO 21338: 2010). An acute test	Most widely used bacterium for ecotoxicity analysis. Numerous toxicity data and QSARS available. Commercially available (Microtox, ToxAlert, *V. fischeri* Reagent, Dr. Lange Lumistox)	30-min EC50>20 000	30-min EC50=1.9	30-min EC50=79
Inhibition of bioluminescence of recombinant bacteria *Escherichia coli*. An acute test	Genome of *E. coli* is sequenced. Popular model organism for evaluation of antibacterial compounds	30-min EC50>20 000	30-min EC50=183	30-min EC50=50.5
	Lowest EC50 (most sensitive organism/test):	9.73 (algae)	0.052 (algae)	0.89 (algae)
	Classification: (7th amendment 92/93 to Directive 67/548/EEC ranks aquatic toxicity of chemicals by the lowest EC50 for the multitrophic test battery (usually daphnids, algae, fish): <1 mg/L: 'very toxic'; 1-10 mg/L : toxic; 10-100 mg/L: harmful)	**Toxic**	**Very toxic**	**Very toxic**

* No specific photoactivation applied for nTiO_2 during testing; ** Toxicity data for algae,[19] crustaceans and Vibrio fischeri,[16,29] protozoa,[28,30] yeast,[26] E. coli AB1157(pSLlux).[24] Data for nTiO_2 toxicity to protozoa are personal communication of Dr. Mortimer (NICPB, Estonia).

FIGURE 4. Characterization of the multitrophic test battery used for the toxicity and environmental hazard ranking of TiO_2, ZnO, and CuO NPs. An experimental case-study. SEM images of nanomaterials are adapted from ref 40. Copyright 2010 Springer.

(protozoa *Tetrahymena*; bacteria *Vibrio fischeri*); and (vi) organisms with genomic data available (*Saccharomyces cerevisiae*, *Tetrahymena thermophila*). Thus, in addition to providing the dose−response data for the regulatory needs, application of the described multitrophic test battery with non-vertebrate ecotoxicological organisms for the first tier toxicity analysis is in accordance with the three R's (Replacement, Reduction, Refinement) strategy that encourages reducing the number of experimental animals for toxicological research.

Our data indicated that, except for algae and to some extent for protozoa, TiO_2 NPs (analyzed without specific photoactivation) showed little or no toxicity even at very high doses applied (Figure 4). The TiO_2 NPs were toxic (72 h EC_{50} = 9.7 mg/L) to algae, presumably due to the entrapment of algal cells by TiO_2 NP agglomerates and subsequent decrease of illumination leading to growth inhibition (Figure 5).[33]

The data showing no adverse effects of TiO_2 NPs to bacterial cells are in agreement with a number of earlier studies.[38] By contrast, Kumar et al. recently showed that TiO_2 NPs were taken up by the *Salmonella typhimurium* bacteria and were mutagenic.[39] Thus, the data on potential hazardous effects of TiO_2 NPs are inconsistent as the current knowledge on the safety of TiO_2 NPs is still emerging.

Compared to TiO_2, ZnO and CuO NPs were remarkably more toxic to all the test organisms used (Figure 4). As one of the first pieces of experimental evidence published, we showed for ZnO NPs in aquatic organisms such as algae *P. subcapitata* or crustaceans *Thamnocephalus platyurus* and *D. magna* that toxicity already became manifested at sub-ppm (<1 mg/L) levels.[31,33] The same also held true for CuO NPs in the case of algae. Thus, in consideration of the fact that *P. subcapitata* and *D. magna* are model organisms for

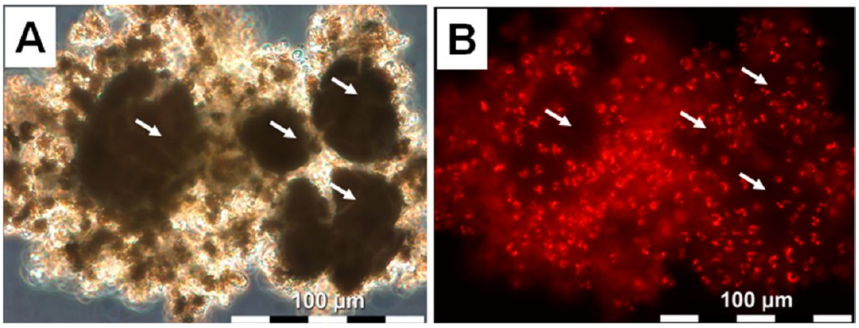


FIGURE 5. *Pseudokirchneriella subcapitata* culture upon exposure to TiO_2 NPs visualized by phase contrast (A) and fluorescence microscopy (B). Arrows indicate algal cells entrapped by nanoTiO_2 aggregates. Notice the absence of planktonic cells in these aggregates in (B). Adapted from ref 33. Copyright 2009 Elsevier.

ecotoxicological analysis of chemicals, these NPs should according to the EU-Directive 93/67/EEC be classified as "very toxic" (Figure 4). It is important to note that, differently from TiO_2, CuO and especially ZnO are sufficiently soluble to release Zn- and Cu-ions in toxic concentrations to a variety of aquatic organisms.[3] Ivask et al. analyzed the solubilization of ZnO and CuO NPs in test media by gene-modified metal-specific biosensors.[40] These recombinant biosensors respond specifically to certain intracellular metal ions by increased bioluminescence[41] and can be applied directly to nanoparticle dispersions without their pre-separation that is methodically a very challenging issue.[42] Using recombinant metal sensor microbes, we showed that the toxicity of ZnO NPs was exclusively explicable by dissolution.[31,33,43,44] Analogously, dissolution of CuO NPs explained its toxic effects to algae and bacteria.[31,33] In addition, our recent study on subtoxic effects of CuO NPs on recombinant *E. coli* bacteria showed that the dissolution of CuO NPs was a key factor triggering various stress responses such as formation of superoxide anions, hydrogen peroxide, and single-stranded DNA.[45] Notably, these effects were observed already at very low subtoxic levels (0.1 mg CuO NPs/L). For some organisms, however, CuO NPs as well as released Cu-ions seemed to possess additional toxic effect. For example, in *D. magna*, upon exposure to subtoxic (48 h NOEC level) concentrations of CuO NPs (but not to bulk CuO or soluble Cu-salts), massive presence of bacteria in the gut was observed that may refer to immune system imbalance even if no internalization of CuO NPs by the gut epithelial cells was observed.[46] Exposure of unicellular eukaryotic organisms *T. thermophila* to CuO NPs (at 24 h EC_{50} level) resulted in changes of membrane fatty acid composition toward increase of rigidity, probably via inhibition of respective desaturases.[47] Thus, the toxicity of metal oxide NPs for aquatic organisms of different biological complexity and feeding strategies may greatly differ (Figure 4). However, due to the paucity of the mechanistic studies on toxicity of metallic NPs toward different environmentally relevant organisms, the specific properties driving the observed toxicity and whether it is a particle or ion-release effect (or both) still have to be investigated.[36]

4.2. A Bibliometrical Case Study from 2009. In an attempt to address the gap in nanoecotoxicological data, a bibliometrical case-study was recently commenced by Kahru and Dubourguier by collecting and summarizing data for seven commercially available NPs: C60, SWCNT, MWCNT, TiO_2, ZnO, CuO, and Ag (Figure 6).[3] The experimental data presented in Figure 4 were also included in this summary. The search focused mainly on aquatic organisms representing main food-chain levels (bacteria, algae, crustaceans, ciliates, and fish). In the case of inorganic NPs, data for respective bulk preparations and respective soluble salts were also included. In addition, the toxicity data for aniline and pentachlorophenol were also collected for the same species "to place" NP toxicities in a known scale. In total, 77 EC_{50} values concerning above-described 7 types of NPs and 7 organism groups were found. Most hazardous to aquatic organisms were Ag and ZnO NPs that to crustaceans (Ag) and algae (ZnO) were toxic already at concentrations below 0.1 mg/L (Figure 6). According to EU-Directive 93/67/EEC, this case study ranked NPs of Ag, ZnO, C60 fullerenes, and CuO as "very toxic" ($EC_{50} < 1$ mg/L); SWCNTs and MWCNTs as "toxic" (EC_{50} = 1−10 mg/L); and TiO_2 NPs as "harmful" (EC_{50} = 10−100 mg/L). Remarkably, none of the NPs studied in the above case study were classified as "unharmful". Moreover, some of these NPs proved as toxic or even more toxic than the well-known biocide pentachlorophenol that has already been banned or severely restricted for health and/or environmental reasons in most countries. The most sensitive organisms toward NPs were algae, followed by crustaceans showing the vulnerability of these organism groups. The fact that in 2009 only 77 EC_{50} values were found for the most

Lowest L(E)C50 value, mg/L	Hazard ranking (EU-Directive 93/67/EEC)	Nanoparticles *(7 altogether) (number of L(E)C50 values found; most sensitive organisms)	Control chemicals (7 altogether) (number of L(E)C50 values found; most sensitive organisms)
<1	Very toxic	•nano-ZnO** (13; algae) •nano-Ag** (10; crustaceans) •C60 (14; ciliates) •nano-CuO (7; algae)	•bulk-ZnO** (13; algae) •Cu^{2+}** (37; algae) •Zn^{2+} (21; algae) •Pentachlorophenol (14; ciliates) •Aniline (13; crustaceans)
1–10	Toxic	•SWCNTs (7; algae) •MWCNTs (2; crustaceans)	None
10–100	Harmful	•nano-TiO_2 (24; algae)	•bulk-TiO_2 (9; algae) •bulk-CuO (6; algae)
Total number of L(EC)50 values analyzed:		77	113
Most sensitive organisms:		Algae (4), crustaceans (2), ciliates (1)	Algae (5), crustaceans (1), ciliates (1)

*All except nCuO belong also to the list of List of Manufactured Nanomaterials for Phase One of the Sponsorship Programme for the Testing of Manufactured Nanomaterials (ENV/JM/MONO(2008)13/REV); ** L(E)C50 < 0.1 mg/L

FIGURE 6. Hazard ranking of seven types of synthetic nanoparticles and seven "not nano" control chemicals based on ecotoxicological data (LC_{50}, EC_{50}) from the scientific literature for selected environmentally relevant test organisms (bacteria, algae, crustaceans, ciliates, fish, and nematodes). Data are summarized from Kahru and Dubourguier.[3] The image is adapted from ref 48.

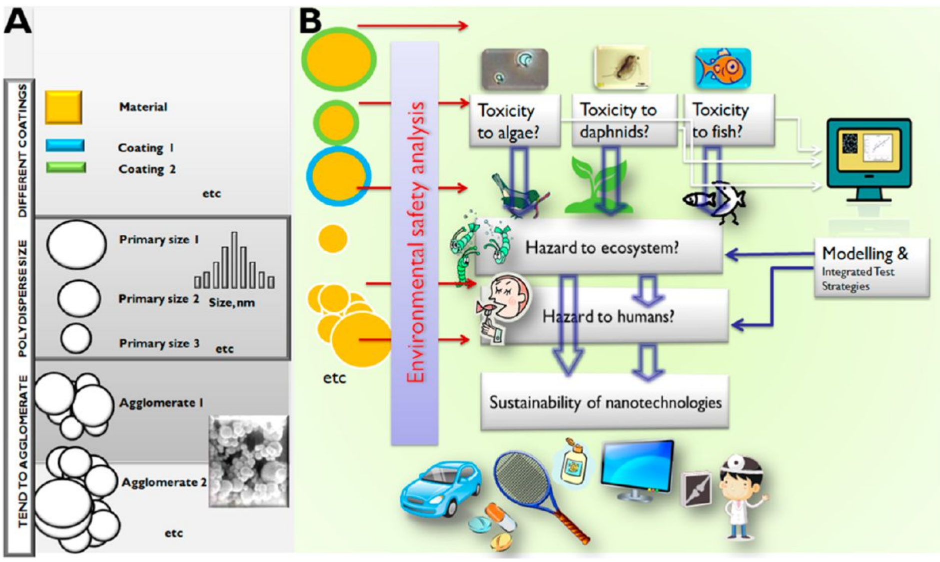


FIGURE 7. Understanding the environmental risks of engineered nanomaterials is a very challenging task. Do we need to test all the possible (nano)particulate entities or we can apply some methodological or conceptual "shortcuts" such as Integrated Test Strategies or computational models? (A) One type of nanomaterial may yield tens to hundreds of (nano)particulate entities with different physical-chemical properties. (B) A crucial step for risk assessment is obtaining toxicity (dose–effect) data for a chemical/substance for a set of environmentally relevant key organisms (algae, daphnids, and fish).

studied NPs and that only 211 papers on search terms "nanoparticles" and "ecotoxic*" were found in November 2011 (Table 1) confirm that nanoecotoxicological research is currently in its infancy.

5. Challenges in the Analysis of Environmental Safety and Ecotoxicological Effects of Nanotechnologies

The holistic understanding of the health and environmental risks of engineered NMs is a very challenging task (Figure 7). Ecotoxicological testing is a part of the environmental risk assessment. As stated above, all chemicals, including nanomaterials, produced in EU by more than 1 metric ton per year need to be ecotoxicologically characterized by 2018. The current information from literature as well as from our own experience shows that analysis of NPs is far more difficult than that of "regular" chemicals, as NP suspensions are unstable, agglomerating in biological media, metallic NPs are often (sparingly) soluble, to mention just some problems that either interfere with test end points or need to be taken into account while testing and drawing conclusions. Moreover, produced NPs are inherently polydisperse, that is, vary in size and often in coating (Figure 7A). Thus, a single NM may actually mean a huge number of combinations of entities with different physicochemical properties which, in turn, could translate into differences in (eco)toxicity (Figure 7) as well as different environmental behaviors of such materials. Therefore, characterization of NPs during (eco)toxicity testing is of vital importance, allowing one to understand how physicochemical properties affect the bioavailability and toxicity.[49] Given these uncertainties, there is a crucial need for cost-effective high throughput methods for (eco)toxicity testing in parallel with physicochemical characterization of NPs.

In addition to testing miniaturization and increase in throughput, computational methods that do not require testing per se have been considered as a powerful alternative to experimental testing in the prediction of potential toxicity and environmental impact of chemicals. The use of quantitative structure–activity relationship (QSAR) methods for predicting the toxic effects of organic chemicals to various organisms is already a relatively common approach also in regulatory risk assessment.[50] For nanomaterials, the QNAR (quantitative nanostructure–activity relationship) models are just emerging. Puzyn et al. showed that a QSAR method reliably predicted the toxicity of 17 different nanometal oxides to *E. coli*.[51] However, QSARs are still of limited use even for "conventional" organic chemicals, mostly due to the lack of good-quality experimental toxicity data.[52,53] Similarly, the lack of quantitative data on toxicological effects of synthetic NPs is limiting the development QNAR models. Moreover, the nano-QSARs are also considerably more difficult than modeling the toxicity of regular chemicals, as respective structural descriptors are significantly more complex (Figure 7A).

6. Outlook

A report "Nano-Regulation" issued at 2005 by various Swiss "nano"-stakeholders objectively stated that *"Regulating an emerging technology is tricky. It is all about striking the right balance between precaution and venture. On one side the protection of health and environmental safety aspects has to be guaranteed and potential risks must be reduced. At the same time, the future social value of nanotechnology should be maximized by all means."*[54] It is obvious that, for the balanced development of nanotechnology, cooperation among industry, scientists, and regulatory agencies is crucial for addressing this complex and important issue of "benefits versus risks" assessment of nanotechnologies.

In this Account, we aimed to provide insight into the emerging scientific information on safety aspects of nanomaterials by focusing on aquatic freshwater species commonly used for risk assessment and regulation. This information can be considered as a starting capital for further accumulation of the scientific knowledge vital for the sustainable coexistence of mankind with increased impact of nanotechnologies.

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FOOTNOTES

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