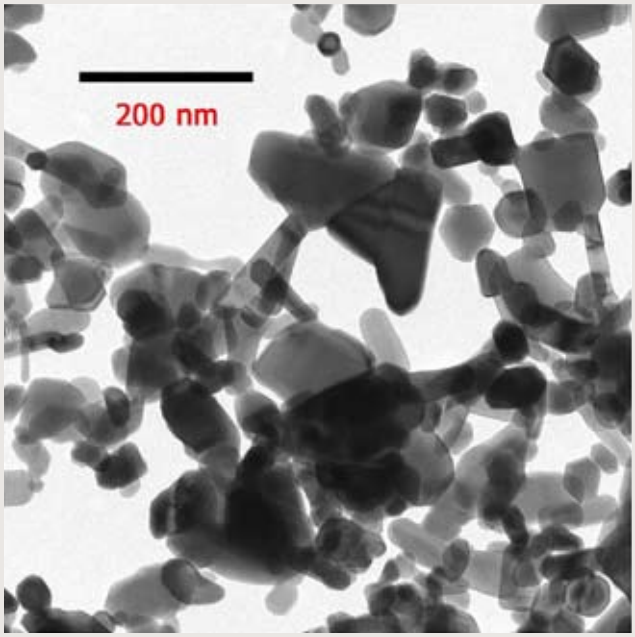
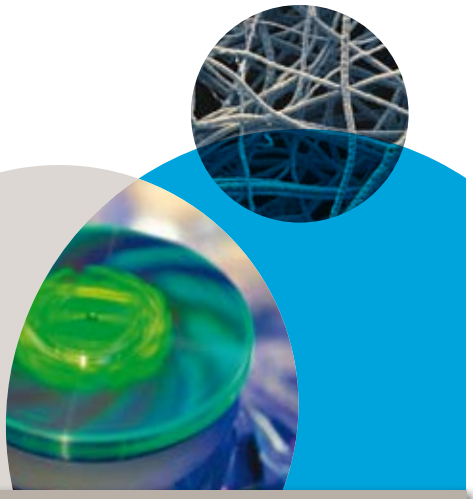
PROSPECT
Global Nanomaterials Safety

# Toxicological Review of Nano Zinc Oxide



8 September 2009

IN SUPPORT OF PROSPECT:

Ecotoxicology Test Protocols for Representative Nanomaterials in Support of the OECD Sponsorship Programme

# Table of Contents

Cover photo: BASF SE Z-COTE® zinc oxide nano particles (Image courtesy of BASF SE)

# FOREWORD/PREFACE

This document is a summary of the literature review on the toxicity and ecotoxicology of nanoparticles in general and nano-zinc oxide in particular. It is to be submitted as the first deliverable of the PROSPEcT Project.

The PROSPEcT Project[‡] is the UK's contribution to the OECD Sponsorship Programme.[§] The PROSPEcT Project aims to examine the environmental safety of nanomaterials in accordance with the agreed OECD WPMN 'Guidance Manual for Sponsors of the OECD Sponsorship Programme for the Testing of Manufacture Nanomaterials'; [1] it will provide crucial data to the OECD work, by addressing gaps in the current level of knowledge on the physico-chemical and ecotoxicological properties of these materials, followed by fundamental scientific research leading to establishing scientific test methodologies to study those endpoints that may not be assessed through standard tests used for bulk chemicals.

The first step of the project consists of a thorough literature review, evaluation exercise, and identification of gaps in the current state of knowledge of the physico-chemical and (eco)toxicological properties of both cerium oxide and zinc oxide nanomaterials. The data was gathered, reviewed and evaluated according to its usefulness to the Project, with specific view to 'addressing' and/or 'completing' the endpoints agreed by the OECD WPMN [1]. This review is crucial to maximise the use of any existing data, to determine what further work needs to be done and to avoid any unnecessary testing.

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[‡] **PROSPEcT: Ec**otoxicology **T**est **P**rotocols for **R**epresentative Nanomaterials in Support of the **O**ECD **S**ponsorship **P**rogramme

[§] OECD's Working Party on Manufactured Nanomaterials (WPMN) launched a Sponsorship Programme in November 2007. The programme involves OECD member countries, as well as some non-member economies and other stakeholders to pool expertise and to fund the safety testing of specific Manufactured Nanomaterials (MNs). In launching this Sponsorship Programme, the WPMN agreed on a priority list of 14 MNs for testing (based on materials which are in, or close to, commerce). They also agreed a list of endpoints for which they should be tested. Much valuable information on the safety of MNs can be derived by testing a representative set for human health and environmental safety.

# 1.0 Introduction

Nanotechnology has gained a great deal of public interest due to the needs and applications of nanomaterials in many areas of human endeavours such as industry, agriculture, business, medicine, public health amongst many others. Nanotechnology includes the integration of these nanoscale structures into larger material components and systems, keeping the control and construction of new and improved materials at the nanoscale. [2]

Nanoparticles can be naturally occurring or they can be manufactured, the latter can be classed into several categories including the following: [3]

1. Metal nanomaterials, such as gold and silver nanoparticles
2. Metal oxide nanomaterials, such as titanium dioxide and zinc oxide
3. Carbon nanomaterials such as fullerenes and nanotubes
4. Quantum dots such as cadmium telluride and cadmium selenide

One estimate for the production of engineered nanomaterials was 2000 tonnes in 2004 and increasing to 58,000 tonnes by 2011-2020. [4]

There are various definitions [5] on nanomaterials in the literature and it should be mentioned that the International Standards Organisation (ISO) has recently developed a technical specification on the terminology and definitions of nano-objects. [6] The specification does not define the term nanomaterial, but it describes the term nano-object as a material with one, two or three external dimensions in the nanoscale, with nanoscale being the size range from approximately 1 nm to 100 nm.** Manufactured nanoparticles often exhibit special physico-chemical properties and reactivities due to their small size and controlled composition, structure or surface characteristics which are not present at the larger scale. In particular, nanoparticles possess a much higher specific surface area (SSA) than their larger counterparts of the same material, and the proportion of atoms on the surface versus the interior of the particle is also much larger. These factors can give rise to higher surface reactivity for the same mass of material. [5]

This literature review consists of a literature summary of the current knowledge on the toxicity and specifically ecotoxicology of nanoparticles in general and nano zinc oxide in particular, followed by an evaluation of the available information with regard to its suitability to be included in the OECD Sponsorship Programme. This summary will be updated periodically to incorporate the latest articles which are thought to be relevant to the project.

Most of the articles used in this review are dispersed across a range of disciplines and focussed primarily on *in vitro* testing and often do not specify an exposure pathway. The literature review search was performed at the end of 2008 using various sites and databases. The Scientific Committee on Consumer Products (SCCP) Opinion as well as the EU-risk assessment on zinc oxide was reviewed if data with nano (ultrafine) zinc oxide were cited. The relevant ecotoxicological and

** NOTE: Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties, size limits are considered approximate. The definition takes into consideration the formation of agglomerates and aggregates, in which nano-objects can be original source particles that are termed primary particles.

toxicological data were compiled and robust study summaries are reported. Ongoing studies as well as studies that are not accessible are not included in the literature review but it may be incorporated in the future if it found to be relevant and available.

Most research is conducted with a focus on the isolated nanomaterials;there is little research on finished consumer products, particularly on their environmental fate. [7] It should be noted however, that most attention has thus far been devoted to the toxicology and health implications of manufactured nanomaterials (MNs) while the behaviour of MN in the environment and their ecotoxicology have been reviewed less. [4]

# 2.0 General Information

## *2.1 Identification*

Reference substance zinc oxide /oxozinc

CAS No: 1314-13-2

EC number: 215-222-5

EC name: zinc oxide

Zinc oxide is an inorganic compound with the formula ZnO. It usually appears as a white powder and is nearly insoluble in water (water solubility of zinc oxide ranges from 1.6 mg/L to 5 mg/L). The powder is used in a variety of applications typically as an additive into products including plastics, ceramics, glass, cement, rubber (e.g. car tyres), lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, etc.

ZnO is present in the Earth crust as a mineral zincite; however most ZnO used commercially is produced synthetically. [8]

# 3.0 Ecotoxicological Information

The relevant ecotoxicological data was compiled and robust study summaries are provided in the Annex 1. Here only a summary is reported.

## *3.1 Aquatic toxicity*

### 3.1.1 Short-term toxicity to fish

Zhu *et al*. compared the toxicity of several metal oxide aqueous suspensions to Zebrafish (Danio rerio) early developmental stage; [9] of the substances tested, ZnO was the most toxic material to zebrafish embryos and larvae, the 96-h LC50 of nano ZnO and ZnO/bulk aqueous suspensions on the zebrafish survival were 1.793 mg/L and 1.550 mg/L, respectively; and the 84-h EC50 on the zebrafish embryo hatching rate were 2.065 mg/L and 2.066 mg/L, respectively. But none of the nano $TiO_z$, $TiO_2$/bulk, nano $Al_2O_3$ and $Al_2O_3$/bulk suspensions showed any significant toxicity to zebrafish embryos and larvae. The zebrafish developmental toxicity of either the nano ZnO or the ZnO/bulk suspensions had an obvious dose-depending property. Metal oxide nanoparticles with different chemical compositions have different zebrafish developmental toxicity. Stability of chemical compositions of nanomaterials itself should be considered as an important factor affecting their potential environmental impacts and biological effects.

### 3.1.2 Short-term toxicity to aquatic invertebrates

Wiench *et al*. reported the acute and chronic effects of nano- and non-nanoscale $TiO_2$ and ZnO particles on mobility and reproduction of the freshwater invertebrate, Daphnia magna. [10] In all acute toxicity tests, the survival in the controls was well above 90%. Hence, the validity criterion as required in the OECD Guideline For Testing Of Chemicals 202 was met.

Toxic effects on Daphnia magna - with EC50 values[††] between 1 and 10 mg/L using M4 medium - were observed in six acute toxicity tests performed with ZnO-based pigments. Differences in effects on daphnids could be found neither using non-nano-scale or nano-scale nor coated or non-coated ZnO pigments. The use of natural water resulted in a decrease of acute toxicity concerning Z-COTE®HP1.

The study concluded that the acute effects of ZnO on the mobility of Daphnia magna are probably due to the ion toxicity of Zn and are not an effect of exposure to the metal oxide ZnO. [10]

Heinlaan *et al.* reported the toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus. [11] All Zn formulations were very toxic: L(E)C50 (mg/L) for bulk ZnO, nano ZnO and $ZnSO_4 \bullet 7H_2O$: 8.8, 3.2, 6.1. The toxicity was due to solubilized Zn ions as proved with recombinant Zn-sensor bacteria.

[††] The term half maximal effective concentration (EC50) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time.

### 3.1.3 Toxicity to aquatic algae and cyanobacteria

Franklin *et al.* compared the toxicity of various metal oxide nanoparticles and zinc chloride to a Freshwater Microalga. [12] The study used nanoparticulate ZnO (ca. 30 nm) showed that small particles in aquatic systems and their bioavailability does not have to be significantly greater than that of larger particles. Particle characterization using transmission electron microscopy and dynamic light scattering techniques showed that particle aggregation is significant in a freshwater system, resulting in flocs ranging from several hundred nanometers to several microns. Chemical investigations using equilibrium dialysis demonstrated rapid dissolution of ZnO nanoparticles in a freshwater medium (pH 7.6), with a saturation solubility in the milligram per litre range, similar to that of bulk ZnO.

Toxicity experiments using the freshwater alga *Pseudokirchneriella subcapitata* revealed comparable toxicity for nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$, with a 72-h IC50 value near 60 µg Zn/L, attributable solely to dissolved zinc. Care therefore needs to be taken in toxicity testing in ascribing toxicity to nanoparticles per se when the effects may be related, at least in part, to simple solubility. [12]

### 3.1.4 Toxicity to microorganisms

Adams *et al*. compared the eco-toxicity of nanoscale $TiO_2$, $SiO_2$, and ZnO water suspensions. [13] Antibacterial activity was reported to increase with dose. The results showed that the Gram-negative E.coli was less sensitive to the addition of ZnO nanoparticles than Gram-positive B. subtilis, which was also tested.

Toxicity was not related to particle size. E. coli was less susceptible to ZnO exposure than B. subtilis, with minimal growth inhibition under dark conditions, which might reflect differences in physiology, metabolism, or degree of contact. [13]

## *3.2 Terrestrial toxicity*

### 3.2.1 Toxicity to soil macroorganisms except arthropods

Unrine *et al.* reported on the spatial distribution and speciation of Au and Zn in terrestrial organisms exposed to Au and ZnO nanoparticles. [14] Patterns of Zn distribution in E. fetida tissues were very similar among Zn treated animals, but the patterns of distribution differed markedly between Zn treated animals and control animals. While Zn treated animals had relatively homogenous distribution of Zn, control animals had loci of extremely high Zn concentrations and they were likely composed of a very high percentage of Zn. These loci produced very intense diffraction patterns indicating that they were composed of crystalline materials. Total Zn concentrations, as determined by acid dissolution and analysis by Inductively coupled plasma mass spectrometry (ICP-MS) were similar in earthworms regardless of treatment. The control earthworms scavenged nearly all of the Zn from the control soil, which contained approximately 20 mg/kg prior to the exposure and non-detectable quantities of Zn after exposure. The distribution of Zn among macromolecules was also similar among Zn-treated earthworms, but some control earthworms had very high concentrations of Zn bound to a ~10 kDa protein, suggesting that large amounts of Zn-metallothionein were present.

The fact that the spatial and molecular distribution of Zn differed between Zn-treated animals and control animals but not among Zn treatments, suggests that Zn from ZnO may have been bioavailable. [14]

The patterns of Zn distribution depended on Zn exposure concentration. Control animals had regions of maximum Zn intensity in the anterior region of the body and these regions of maximal intensity spread towards the anterior end of the body with increasing exposure concentration from 100 to 500 mg Zn/L. The maximum Zn Kα intensities in the $ZnCl_2$ exposed nematodes were nearly twice as great as in the ZnO exposed nematodes suggesting decreased bioavailability of Zn as ZnO relative to aqueous Zn ions. The fact that the LC50 values of these two forms of Zn were similar despite decreased bioavailability of Zn in nano-ZnO form, suggests that the two forms of Zn elicit toxicity by differing mechanisms. The regions of high Zn intensity did not produce diffraction patterns for any treatment; however, if crystalline materials were present, a detectable diffraction pattern would not be expected at such low concentrations. [14]

### 3.2.2 Toxicity to terrestrial arthropods

Wan *et al.* studied the effect of action of mixture of two nano particles with two insecticides to pest mite (Epitrimerus pyri). [15]According to the authors the actives Cypermethrin and alpha Terhienyl mixed with nano-particled zinc oxide and copper oxide showed syntergistic effects to the tested mite.

### 3.2.3 Toxicity to terrestrial plants

Lin studied the effect of phytotoxicity of nanoparticles and its effect on seed germination and root growth. [16] Seed germinations were not affected by the nanoparticles except for seeds of ryegrass and corn. Seed germination of ryegrass and corn was inhibited by nano-Zn and nano-ZnO, respectively. The influence of nanoparticle suspensions at 2000 mg/L on root growth varied with types of nanoparticles and plant species. Phytotoxicity of nano-Zn and nano-ZnO was evident. Their suspensions significantly inhibited root growth of corn and practically terminated root development of the other five plant species whose root length at the end of experiment was unable to be accurately measured with a ruler. However, they all germinated with cotyledons sprouting out of seed coat.

To examine which process (seed soaking or incubation after the soaking) primarily retarded the root growth, three treatments were used: (1) both seed soaking and incubation were performed in nanoparticle suspensions; (2) seeds were soaked in nanoparticle suspensions for 2 h, and were then transferred into Petri dishes with 5 mL DI-water for incubation after being rinsed three times with DI-water; and (3) seeds were incubated in Petri dishes with 5 mL nanoparticle suspensions after being soaked in DI-water for 2 h. As described, the root growth was almost halted by seed soaking and incubation in the suspensions of nano-Zn and nano-ZnO (the first treatment). Also, root growth of radish, rape and ryegrass was nearly terminated under the third treatment (soaking in water, then incubation in suspension), while roots grew relatively well under the second treatment (soaking in suspension, then incubation in water) though the root development of the three plant species was significantly inhibited by nano-Zn, and, that of ryegrass by nano-ZnO. [16]

To further clarify the phytotoxicity of nano-Zn and nano-ZnO, experiments were carried out to determine the dose-response relationship of nano-Zn and nano-ZnO using the first treatment: both seed soaking and incubation in the nanoparticle suspensions or $Zn^{2+}$ solutions. No significant root growth inhibition was observed under low concentrations (less than 10 mg/L for rape and ryegrass and 20 mg/L for radish). Root growth was clearly restricted with increasing concentration, and was almost terminated at 200 mg/L. Nano-Zn and nano-ZnO showed similar phytotoxicity at each of concentrations. Fifty percent root growth inhibitory concentrations (IC50) of both nano-Zn and nano-ZnO were estimated to be near 50 mg/L for radish, and near 20 mg/L for rape and ryegrass.

Two experiments were conducted to exclude $Zn^{2+}$ from the phytotoxicity of nano-Zn and nano-ZnO. First, the phytotoxicity of supernatants of the nanoparticle suspensions after centrifuging at 3000 g for 1 h and filtering through 0.7 µm glass filters was measured, then the phytotoxicity of $Zn^{2+}$ solutions by dissolving $ZnSO_4 \bullet 7H_2O$ in DI-water was tested. No statistically significant effect (negative or positive) was observed except for the growth enhancement by the supernatant of ZnO suspension at 2000 mg/L on rape ($p = 0.050$) and radish ($p = 0.014$). Concentrations of $Zn^{2+}$ in the supernatants (after centrifugation and filtration) were 0.3-3.6 mg/L. Therefore, five concentration points of 0, 1, 2, 3 and 4 $Zn^{2+}$ mg/L were made from $ZnSO_4 \bullet 7H_2O$ to investigate the phytotoxicity of $Zn^{2+}$. No significant effect on the root of radish, rape and ryegrass from these $Zn^{2+}$ concentrations was observed. [16]

Lin also reported the phytotoxicity effect of ZnO nanoparticle on ryegrass growth. [17] Toxicity of nano-ZnO on ryegrass was obvious. The growth of seedlings was greatly inhibited under the nano-ZnO treatments. Ryegrass shoot biomass significantly (p0.05) reduced with increasing concentration of nano-ZnO in the nutrient solution, especially at the nano-ZnO concentrations higher than 20 mg/L. The shoot growth inhibition by the nano-ZnO was worst at 200 mg/L, with nearly constant biomass at concentrations up to 1,000 mg/L. Biomass reduction of ryegrass root showed a similar tendency. But significant root inhibition was observed only at nano-ZnO concentrations of 100-1,000 mg/L and the root biomass kept nearly unchanged in this concentration range. No significant difference of the ryegrass biomass between the treatments of $Zn^{2+}$ and nano-ZnO could be observed. But the yellow and withered shoots at higher $Zn^{2+}$ concentrations indicates that $Zn^{2+}$ might be more toxic to the ryegrass than nano-ZnO. It was concluded, the phytotoxicity of nano-ZnO could not mainly come from its dissolution at root surface or inside root tissue.

Root uptake and phytotoxicity of ZnO nanoparticles was also reported by Lin. [18] The toxicity of Zn0 nanoparticles and $Zn^{2+}$ ions to the ryegrass seedlings were evident and increased with increasing concentration of both ZnO nanoparticles and $Zn^{2+}$, which could be easily observed by visual examination. The growth of seedlings in both treatments, especially with concentrations higher than 50 mg/L, was retarded with shorter roots and shoots than the control. Toxic symptoms seem more severe with $Zn^{2+}$ than ZnO nanoparticles, in that shoots became yellow with concentrations of $Zn^{2+}$ higher than 50 mg/L and almost withered to death at a concentration up to 1000 mg/L. As regards the dose-response curves of ZnO nanoparticles and $Zn^{2+}$, there appeared a concentration threshold of both treatments, below which no significant toxic symptoms were observed. However, the seedling biomass decreased with increasing dose after the threshold. The threshold of $Zn^{2+}$ for both shoot and root was ca. 20 mg/L, whereas it was around 10 and 50 mg/L of ZnO nanoparticles for ryegrass shoots and roots, respectively. SEM images showed that root surface in the control and $Zn^{2+}$

treatments were free of particle adherence. However, adsorption of ZnO nanoparticles and their aggregations on the root surface was evident and the coverage increased with increasing ZnO dose.

The particles were observed filled in the epidermal crypt or adhered onto the surface. TEM images of the cross sections of the ryegrass roots show the presence of dark dots (particles) in the endodermis and vascular cylinder under the ZnO treatments. The dark dots were distributed in the apoplast, cytoplasm, and even nuclei. One or several nanoparticles could be identified in the dark dots covered by cytoplast as shown by higher magnification TEM image. The size of these nanoparticles were measured to be 19 ± 6 nm (n = 89), identical to the size of ZnO nanoparticles. Such dark dots (i.e. particles) were not observed under either the control or $Zn^{2+}$ treatments. Thus, it was concluded that the ZnO nanoparticles could enter into the endodermis and vascular cylinder of the ryegrass roots. In summary the authors concluded, ZnO nanoparticles were found able to concentrate in the rhizosphere, enter the root cells, and inhibit seedling growth; the phytotoxicity of ZnO nanoparticles could not primarily come from their dissolution in the bulk nutrient solution or the rhizosphere.

# 4 Toxicological information

The relevant toxicological data was compiled and robust study summaries are provided in the Annex 2. Here only a summary is reported.

## *4.1 Toxicokinetics, metabolism and distribution*

### 4.1.1 Dermal absorption

Gamer and Nohynek reported on the dermal adsorption of zinc oxide particles. [19,20,21] The amount of Zn applied to the skin preparations as a ZnO containing formulation was quantitatively removed with the first 5 tape strips, representing mainly the test substance film on the skin. There was no increase of Zn in the tape stripped skin preparation or in the receptor fluid. Thus no Zn from the ZnO containing formulation penetrated into or through the skin of domestic pigs under the conditions of this study. In addition the results show that microfine ZnO particles were not able to penetrate the porcine dermatomed skin preparations.

Cross *et al.* reported on human skin penetration of sunscreen nanoparticles. [22] Human epidermal penetration of a novel, transparent, nanoparticulate zinc oxide sunscreen formulation was determined using Franz-type diffusion cells, 24-hour exposure and an electron microscopy to verify the location of nanoparticles in exposed membranes. Less than 0.03% of the applied zinc content penetrated the epidermis (not significantly more than the zinc detected in receptor phase following application of a placebo formulation). No particles could be detected in the lower stratum corneum or viable epidermis by electron microscopy, suggesting that minimal nanoparticle penetration occurs through the human epidermis.

Zvyagin *et al.* investigated the distribution of topically applied ZnO in excised and *in vivo* human skin, using multi photon microscopy (MPM) imaging with a combination of scanning electron microscopy (SEM) and an energy-dispersive x-ray (EDX) technique to determine the level of penetration of nanoparticles into the sub-dermal layers of the skin. [23]

The overall outcome from MPM, SEM and EDX studies was that, in humans *in vivo* ZnO nanoparticles stayed in the stratum corneum and accumulated into skin folds and/or hair follicle roots of human skin. Given the lack of penetration of these nanoparticles past the stratum corneum and that the outermost layers of the stratum corneum have a good turnover rate, the data suggest that the ZnO-nano form studied here is unlikely to result in safety concerns.

## *4.2 Acute Toxicity*

### 4.2.1 Acute toxicity: oral

*Wang et al.* studied the acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice. [24] Combined with the results of zinc accumulation, pathological examination and the biological indicators assays, the target organs for 20- and 120-nm ZnO acute oral administration are demonstrated to be the liver, heart, spleen, pancreas and bone.

The biochemical and pathological investigation shows that the toxic effects between the 20 nm and 120 nm ZnO particles are a little different. For example, the blood viscosity could be induced by low and median dose of 20 nm ZnO but high dose of fine ZnO after oral administration. The edema and degeneration of hepatocytes, and inflammation of pancreas could be observed in most of the 20 nm ZnO treated mice. The 120 nm ZnO treated mice were found having dose-effect pathological damage in gastric, liver, heart and spleen, however, the 20 nm ZnO treated mice presented lessened liver, spleen and pancreas damage with the increase of treated dose.

The relevance of the study is unclear - how these effects are due to the size. The study concludes that the zinc oxide particles are nontoxic.

### 4.2.2 Acute toxicity: inhalation

Sayes *et al*. assessed the toxicity of fine and nano-scale particles. [25] The study concluded the following.

***In vivo* Bronchoalveolar lavage (BAL) Fluid LDH Response:**

Exposure to nano-ZnO or fine-ZnO particles suspensions produce enhanced cytotoxic responses at 24 h and 1 week post instillation exposure (pe) time periods but were not different from PBS-instilled controls thereafter.

***In vivo* BAL Fluid Total Cell Numbers:**

The number of cells at the 24 h time point for rats exposed to nano-ZnO and fine-ZnO were significantly higher than the other time points. This increase is a transient effect due to the instillation procedure and was diminished at the 1 week time point.

***In vivo* Pulmonary Inflammation**

Intratracheal instillation exposure to high-dose nano-ZnO (5 mg/kg) or fine-ZnO particles produced substantial lung inflammatory responses measured at 24 h pe followed by a minimal, recruitment of neutrophils through 1 week pe (i.e. 15 -20 % polymorphnuclear leukocytes). These effects were not measured at the 1 and 3 month pe time points, indicating resolution of the inflammatory responses.

### 4.2.3 Acute toxicity: other routes

Liu *et al.* reported on the Acute Toxicity of Nano-sized Zinc Oxide (N-ZnO) in ICR Mice via Intratracheal Instillation. [26] Histopathological observation showed that serious pulmonary inflammation, proliferation and thickening of alveolar walls existed in the lungs of all treated mice groups, which became more serious as the dose increased.

The intratracheal instillation of N-ZnO induced significant pulmonary inflammation and marked body weight loss accompanied with anemia.

## *4.3 Repeated dose toxicity*

### 4.3.1 Repeated dose toxicity: inhalation

Ma-Hock *et al.* reported on the repeated dose toxicity of nano-scale zinc oxide and pigmentary zinc oxide by inhalation. [27,28] The inhalation of Z-COTE HP1 for 5 days resulted in local inflammations in the lungs of the rats, indicated by changes in several parameters in the bronchoalveolar lavage fluid (BALF) and histological examinations. Secondary to the effect in the lung, activation of the draining lymph nodes was noted. Moreover, minimal to moderate necrosis of the olfactory epithelium was noted. The effects were in a concentration-related manner and were reversible within the recovery period. Only a multifocal increase in alveolar macrophages was still present at the end of the recovery period. Similar effects were also observed in the animals exposed to ZnO powder.

At the low concentration of 0.55 mg/$m^3$, increased levels of a few mediators in the BALF and in serum were determined. Moreover minimal multifocal necrosis of the olfactory epithelium of grade was noted in the nasal cavity in one of the six animals. Therefore, a No Observed Adverse Effect Concentration (NOAEC) could not be established in this study. The lowest concentration of 0.55 mg/$m^3$ is considered to be the Low Observed Adverse Effect Concentration (LOAEC).

Several other studies have also been reported on this subject. [29,30,31,32,33] Typically, after inhalation exposure studies of short duration (3-6 days) are available. In a 3-day inhalation study with guinea pigs a concentration of 2.3 mg ultra fine ZnO/$m^3$ (3 hours/day) was a marginal LOAEL, showing changes in neutrophils and activities of lactate dehydrogenase and alkaline phosphatase in the pulmonary fluid. At higher concentrations increased protein concentration, neutrophils, and enzyme activities in lung lavage fluids were seen, together with significant centriacinar inflammation of the pulmonary tissue. A dose of 2.7 mg ultra fine ZnO/$m^3$ (3 hours/day for 5 days) did not alter the lung function parameters in guinea pigs but at 7 mg ultra fine ZnO/$m^3$ (3 hours/day for 5 days) or at 5 mg ultra fine ZnO/$m^3$ (3 hours/day for 6 days) a gradual decrease in total lung capacity, vital capacity and reduction of carbon monoxide diffusing capacity were seen in combination with inflammatory changes and edema. The relevance of the findings in studies with ultra-fine zinc oxide fumes is unclear with respect to commercial grade zinc oxide, as the latter is of much larger particle size and can have different toxicological characteristics.

## *4.4 Genetic toxicity*

### 4.4.1 Genetic toxicity *in vitro*

Salmonella typhimurium reverse mutation assays according to OECD TG 471 were conducted by BASF on the Z-COTE HP1 and Z-COTE MAX material [34,35], showing no mutagenic effect in this test-system.

### 4.4.2 Genetic toxicity *in vivo*

BASF also studied the effect of Z-COTE HP1 in the *in vivo* micronucleus test in bone marrow cells of mouse (OECD TG 474). [36] Under the experimental conditions chosen, the test substance Z-COTE HP1 does not have any chromosome damaging (clastogenic) effect, and there were no indications of any

impairment of chromosome distribution in the course of mitosis (aneugenic activity) in bone marrow cells *in vivo*.

## *4.5 Specific investigations*

### 4.5.1 Specific investigations: other studies

A couple of specific studies were done on the phototoxicity of zinc oxide. [37,38] Diambeck studied the effect of Zinc Oxide (H&R, PN 104702) using the 3T3 Neutral Red Uptake Phototoxicity Test. [37] The studies concluded that both ZnO irradiated and unirradiated had similar cytotoxic concentration response curves. Both curves were similarly shaped with nearly identical NR50 values at approximately 9 mg/L. The ratio of [NR50 (-UV)/ NR50 + UV)] PIF equals 1, demonstrating that ZnO does not exhibit a phototoxic potential in this test.

Dufour *et al.* studied the photoclastogenicity of zinc oxide. [39] The results provide evidence that, under conditions of *in vitro* photo-clastogenicity tests, UV irradiation of the cellular test system per se may produce a slight increase in the genotoxic potency of compounds that are clastogenic in the dark. In conclusion, the data suggest that minor increases in clastogenic potency under conditions of photo-genotoxicity testing do not necessarily represent a photo-genotoxic effect, but may occur due to an increased sensitivity of the test system subsequent to UV irradiation.

## *4.6 Exposure related observations in humans*

### 4.6.1 Sensitisation data (humans)

There is some sensitisation data performed on humans. [38,40] Both Studies conclude that ZnO as tested, was neither a skin irritant nor a skin sensitiser.

### 4.6.2 Exposure related observations in humans: other data

**Inhalation**

There is some inhalation data reported by Kuschner *et al.* and the European Chemicals Bureau. [41,42] Metal fume fever is a flu-like illness caused by zinc oxide fume inhalation and mediated by unknown mechanisms. It is one of a group of work-related febrile inhalational syndromes.

The bronchoalveolar lavage (BAL) obtained from cigarette smoking and non smoking human volunteers was examined after controlled exposure to purified zinc oxide fume to explore the possible roles of proinflammatory cytokines in this condition.

Purified zinc oxide fume inhalation caused an exposure-dependent increase in proinflammatory cytokines and Polymorphonuclear leukocytes (PMNs) in the lung.

**Photoirritation**

Several studies have also reported data on the photoirritation caused by zinc oxide particles. [38,43,44] The studies conclude that no phototirritant skin reaction in any volunteer was observed, thus ZnO as tested was not photo-irritant.

## *4.7 Additional toxicological information*

**Toxicity to mammalian cells**

Jeng *et al.* studied the toxicity of metal oxide nanoparticles in mammalian cells. [45] Dramatic changes in the cell morphology were observed after exposure to ZnO for 24 hours, particularly at concentrations greater than 50 µg/mL. The cells became irregular and shrank. At the concentration of 50 µg/mL, some cells retained an intact plasma membrane indicating that apoptosis occurred. At the concentration of 100 µg/mL, they became necrotic and detached from the culture dishes. At concentrations below 10 µg/mL, no distinct change in morphology of cells was observed.

At concentrations of 50 to 100 µg/mL, ZnO nanoparticles caused 15% to 50% of the cells to die as detected by the trypan blue dye method. However, cell viability was not affected by ZnO exposure at a concentration of less than 25 µg/mL for 48 hours.

The mitochondrial function (MTT) results showed that ZnO exhibited more toxicity than other metal oxide nanoparticles. A dose-dependent increase in reduction of mitochondrial function was observed with ZnO exposure. After 24 hours of exposure, ZnO at a concentration of 100 µg/mL reduced the mitochondrial function >80% as compared to the control cells. At a concentration of 10 µg/mL, ZnO showed insignificant effects on the mitochondrial function.

A significant increase in LDH leakage was observed with exposure to ZnO. At 4 hours of incubation, cells treated by ZnO at a concentration of 50 µg/mL started showing a decrease in mitochondrial function and LDH leakage. At lower concentrations, the effect appeared after 6 hours of contact time.

**Toxicity to bacteria**

Mashayekhi *et al.* studied the effect of metal oxide nanoparticles on bacteria. [46] All nanoparticles except $TiO_2$ showed toxicity to bacteria significantly more than their micro counterparts. Nano $TiO_2$ toxicity to bacteria is reported at higher concentrations (1,000 mg/L, Adams *et al.* [13]) than what was used in this study (20 mg/L). Gram positive and Gram negative bacteria did not behave differently to nanoparticle toxicity. The order of observed toxicity to nanoparticles in E.coli was $Al_2O_3 < SiO_2 > ZnO$ Different bacteria had varied tolerance to nanoparticles. TEM images confirmed attachment of nanoparticles to the bacteria. Observed toxicity of nanoparticles was not due to the free metal ions dissolved from metal oxides. It seems that size of particles plays an important role in their toxicity.

Brayner *et al.* also performed similar studies on bacteria: [47] E. coli cells, after contact with di(ethylene glycol) (DEG) and ZnO, were damaged showing a Gram-negative triple membrane disorganization. This behaviour causes the increase of membrane permeability leading to accumulation of ZnO nanoparticles in the bacterial membrane and also cellular internalization of these nanoparticles. Lower concentrations of ZnO nanoparticles did not induce any damage E. coli cells after contact with ZnO nanoparticle concentrations higher than $1.3 \times 10^{-3}$M and with some small molecules and macromolecules in DEG medium were damaged and the cell contents may have leaked out. Cellular internalization of these nanoparticles was observed.

Hernandez-Sierra *et al.* also reported on the toxicity to bacteria: [48] for silver the results showed an average minimum inhibitory concentrations (MIC) of 4.86 ± 2.71µg/mL and minimum bactericidal concentrations (MBC) of 6.25µg/mL; for zinc the MIC was 500 ± 306.18µg/mL and MBC of 500µg/mL; the gold nanoparticles demonstrated an effect only at an initial concentration of 197µg/mL. A higher antimicrobial effect against S. mutans of silver nanoparticles at lower concentrations than gold or zinc was established.

**Antibacterial activity**

Jones *et al.* reported on the antibacterial activity of ZnO nanoparticle suspension on a broad spectrum of microorganisms. [49] The studies concluded the following:-

Among the nanoparticles tested, four nanoparticles (MgO, $TiO_2$, CuO and $CeO_2$, did not show any significant growth inhibition up to 10 mM colloidal suspension, whereas $Al_2O_3$(c. 50%) and ZnO (>/= 50%) showed significant growth inhibition. ZnO nanoparticles showed a significant growth inhibition compared with the control. No significant growth inhibition was detected with the used experimental setting for Staphylococcus aureus.

For ZnO ultrafine powder and ZnO nanopowder, which have relatively large particle sizes, reduced growth rates (ca. 50%) were observed. However, ZnO nanoparticles, with smaller particle size, were able to reduce ca. 99% of growth colloidal suspension concentration of 2mM.

The data for both sizes of ZnO nanoparticles clearly suggest that the antibacterial activity of ZnO nanoparticles in the dark is less than that in ambient laboratory conditions and the authors concluded that the ambient laboratory conditions are sufficient for the optimal biocidal activity of the ZnO nanoparticles, which is probably dependent on the size of the nanoparticles.

Zhang *et al.* also performed a similar study using ZnO nanofluids. [50] The results show that the ZnO nanofluids have bacteriostatic activity against E. coli. The antibacterial activity increases with increasing nanoparticle concentration and increases with decreasing particle size. Particle concentration is observed to be more important than particle size under the conditions of this work. The results also show that the use of two types of dispersants, Polyethylene Glycol (PEG) and Polyvinylpyrolidone (PVP), does not affect much the antibacterial activity of ZnO nanofluids but enhances the stability of the suspensions. SEM analyses of the bacteria before and after treatment with ZnO nanofluids show that the presence of ZnO nanoparticles damages the membrane wall of the bacteria. Electrochemical measurements using a model DOPC monolayer suggest some direct interaction between ZnO nanoparticles and the bacteria membrane at high ZnO concentrations.

# 5.0 Relevance to the OECD Sponsorship Programme.

The OECD Sponsorship Programme is valuable and relevant information on the safety of MNs and the methods used to assess their safety can be derived by appropriately testing a representative set of MNs for identified human health and environmental safety endpoints. The Working Party for Manufactured Nanomaterials (WPMN) has determined that the programme is intended to develop the data that will improve the understanding of MNs, and, if possible, to understand what information may be generalised across different MNs or classes of MN. [1]

The programme contains two phases:

**Phase 1**

In Phase 1, the WPMN has invited WPMN participants to volunteer to sponsor the testing of one or more of the MNs on the list of representative MN. Sponsors ‡‡ have been asked to prepare a Dossier Development Plan (DDP) for the testing of the MN. The DDP will be reviewed by the WPMN. Based upon the reviewed DDP, Sponsors would complete Phase 1 by addressing those endpoints (listed below) appropriate for the material. The scope of Phase 1 is to provide a dataset by addressing the endpoints listed below. This includes, where appropriate, the utilisation of existing relevant information, the generation of new information or the rational why the information is not needed. Also where specified in the Guidance Manual, 'address' §§ includes the term 'completed', *** which provides that all dossiers will contain the identified endpoint information. [1]

Where it is not feasible or not appropriate to develop test data for an endpoint, a rationale for not testing must be provided. Phase 1 is exploratory in nature as the testing methodology and strategy may need to be developed and might evolve during Phase 1 testing. As far as possible and appropriate, a full dataset shall be generated for each MN independent from decision logic based on risk management considerations. The sum of datasets generated by Phase 1 testing together with the methodology developed and experiences gained will help to understand properties specific to the nanoscale features of MNs and to identify data to be developed in Phase 2 testing.

**Phase 2**

The scope of Phase 2 is to address additional endpoints that are necessary to gain understanding of the hazard potential of the respective sponsored MN. The combined data provided by Phase 1 and Phase 2 testing will allow, but not necessarily be entirely sufficient for, application to risk assessment paradigms as considered for specific sponsored MN applications (given that adequate exposure data are available). Phase 2 would also provide an opportunity to consider aspects as life cycle of MNs and evaluation of by-products of the use of nanomaterials in greater depth and specificity than may be possible in Phase 1. Thus, Phase 2 testing may be guided by risk-related decision logic.

‡‡ A Sponsor assumes responsibility for conducting or coordinating all of the testing determined to be appropriate and feasible to address the endpoints of Phase 1 for a listed MN.

§§ Possible ways to 'address' an endpoint are: 'not relevant', 'not measurable', 'read-across' and 'data provided'.

*** Data must be provided for endpoints indicated as 'this element must be completed' (*i.e.* 'completed' = 'data provided')

## *5.1 Endpoints for testing nanomaterials*

| | Status (to be completed/addressed) | Input from Literature |
|---|---|---|
| ***Nanomaterial Information/Identification*** | | |
| • *Nanomaterial name* | All endpoints must be completed | Yes |
| • *CAS Number* | | Yes |
| • *Structural formula/molecular structure* | | Yes |
| • *Composition of nanomaterial being tested (including degree of purity, known impurities or additives)* | | Yes |
| • *Basic morphology* | | Yes |
| • *Description of surface chemistry (e.g., coating or modification)* | | Yes |
| • *Major commercial uses* | | Yes |
| • *Known catalytic activity* | | Yes |
| • *Method of production (e.g., precipitation, gas phase)* | | Yes |
| ***Physical-chemical Properties and Material Characterization*** | | |
| • *Agglomeration/aggregation* | Must be Addressed | Some data available |
| • *Water Solubility/Dispersibility* | Must be Completed | Some data available |
| • *Crystalline phase* | Must be Completed | Some data available |
| • *Dustiness* | Must be Addressed | Some data available |
| • *Crystallite size* | Must be Addressed | Yes |
| • *Representative Electron Microscopy (TEM) picture(s)* | Must be Addressed | Yes |
| • *Particle size distribution – dry and in relevant media* | Must be Completed | Yes |
| • *Specific surface area* | Must be Completed | Yes |
| • *Zeta potential (surface charge)* | Must be Completed | None |
| • *Surface chemistry* | Must be Completed | Yes |
| • *Photocatalytic activity* | Must be Addressed | Some data available |
| • *Pour density* | Must be Addressed | None |
| • *Porosity* | Must be Addressed | None |
| • *Octanol-water partition coefficient* | Must be Addressed | Not relevant |
| • *Redox potential* | Must be Addressed | None |
| • *Radical formation potential* | Must be Addressed | None |
| • *Other relevant Physical-Chemical Properties and Material Characterization information (where available)* | Must be Addressed | None |
| ***Environmental Fate*** | | |
| • *Dispersion stability in water* | Must be Addressed | None |
| • *Biotic degradability* | Must be Addressed | Not relevant |
| • *Identification of degradation product(s)* | Must be Addressed | Not relevant |
| • *Further testing of degradation product(s) as required* | Must be Addressed | Not relevant |
| • *Abiotic Degradability and Fate* | Must be Addressed | Not relevant |
| • *Adsorption-Desorption* | Must be Addressed | None |
| • *Adsorption to soil or sediment* | Must be Addressed | None |
| • *Bioaccumulation potential* | Must be Addressed | None |
| • *Other relevant environmental fate* | Must be Addressed | None |

| | | |
|---|---|---|
| *information (when available)* | | |
| ***Environmental Toxicology*** | | |
| • *Effects on pelagic species (short term/long term)* | Must be Addressed | None |
| • *Effects on sediment species (short term/long term)* | Must be Addressed | None |
| • *Effects on soil species (short term/long term)* | Must be Addressed | Yes |
| • *Effects on terrestrial species* | Must be Addressed | None |
| • *Effects on microorganisms* | Must be Addressed | Yes |
| • *Effects on activated sludge at WWTP* | Must be Addressed | None |
| • *Other relevant information (when available)* | Must be Addressed | None |
| ***Mammalian Toxicology*** | | |
| • *Pharmacokinetics/Toxicokinetics (ADME)* | Must be Addressed | None |
| • *Acute toxicity* | Must be Addressed | Yes |
| • *Repeated dose toxicity* | Must be Addressed | Yes |
| • *Chronic toxicity* | Must be Addressed | None |
| • *Reproductive toxicity* | Must be Addressed | None |
| • *Developmental toxicity* | Must be Addressed | None |
| • *Genetic toxicity* | Must be Addressed | Yes |
| • *Experience with human exposure* | Must be Addressed | Yes |
| • *Other relevant test data* | Must be Addressed | Yes |
| ***Material Safety***<br>• *Flammability*<br>• *Explosivity*<br>• *Incompatibility* | | |

Even though some data is available through the literature, major gaps exist in the list of endpoints being tested. Due to different nanomaterial samples that are being investigated for PROSPEcT and the Sponsorship Programme, it will be necessary to perform most of the listed endpoints where ever applicable.

# 6.0 Conclusion

(Eco)toxicology testing of engineered nanoparticles requires the development of agreed testing protocols and guidelines to allow for the comparison and interpretation of data from the studies. In order to achieve more comparable and reproducible data, reference materials need to be agreed upon, and standards may have to be developed. [51] However, it is still not clear, at which concentrations and in what form ENPs will be released into the environment and so it is difficult to devise experiments to simulate what might happen when nanoparticles are released into soil or the aquatic environment. It is extremely important therefore to establish and agree the criteria for such tests at the beginning of this project.

This literature review illustrates that the existing literature on nano cerium oxide does not provide the required level of comparability and reproducibility for any of the endpoints agreed by the OECD WPMN Sponsorship Programme. In particular the use of the same nanomaterial from the same batch, stored under controlled conditions and tested under agreed sample-handling protocol represent important steps in obtaining viable data on nano cerium oxide, and making this data ultimately comparable to the data obtained from the other nanomaterials within the OECD WPMN Sponsorship Programme.

# References


[1] GUIDANCE MANUAL FOR THE TESTING OF MANUFACTURED NANOMATERIALS: OECD's SPONSORSHIP PROGRAMME., Available at http://www.olis.oecd.org/olis/2009doc.nsf/linkto/env-jm-mono(2009)20

[2] Ju-Nam, Y *et al.; Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications.* Sci Total Environ., 2008, 400(1-3): pg 396-414.

[3] Chandra Ray, P *et al.; Toxicity and Environmental Risks of Nanomaterials: Challenges and Future Needs*. Journal of Environmental Science and Health, Part C, Vol 27, Issue 1, 2009 , pg 1 – 35.

[4] Nowack, B *et al.; Occurrence, behavior and effects of nanoparticles in the environment*. Environmental Pollution, Vol 150, Issue 1, 2007, Pg 5-22.

[5] Handy, RD *et al.; The ecotoxicology and chemistry of manufactured nanoparticles*. Ecotoxicology, 2008, Vol 17, Number 4, pg 287-314.

[6] ISO/TS 27687:2008 Nanotechnologies -- Terminology and definitions for nano-objects -- Nanoparticle, nanofibre and nanoplate.

[7] Ostrowski, AD et al; *Nanotoxicology: characterizing the scientific literature, 2000–2007.* Journal of Nanoparticle Research, 2009, Vol 11, Number 2, pg 251-257.

[8] http://en.wikipedia.org/wiki/Zinc_oxide

[9] Zhu X *et al.*, *Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (Danio rerio) early developmental stage*, J Environ Sci Health A Tox Hazard Subst Environ Eng., 2008, Feb 15; 43(3):278-84.

[10] Wiench K *et al.*, *Acute and chronic effects of nano- and non-nanoscale TiO2 and ZnO particles on mobility and reproduction of the freshwater invertebrate Daphnia magna*, submitted to Chemosphere, 2009.

[11] Heinlaan M *et al., Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus*, Chemosphere, Volume 71, Issue 7, April 2008, Pages 1308-1316.

[12] Franklin, NM *et al., Comparative Toxicity of Nanoparticulate ZnO, Bulk ZnO, and $ZnCl_2$ to a Freshwater Microalga (Pseudokirchneriella subcapitata): The Importance of Particle Solubility,* Environ. Sci. Technol., 2007, 41 (24), pp 8484–8490.

[13] Adams, LK *et al., Comparative eco-toxicity of nanoscale $TiO_2$, $SiO_2$, and ZnO water suspensions*, Water Research, Volume 40, Issue 19, November 2006, Pages 3527-3532.

[14] Unrine, J. *et al.*, *Spatial distribution and speciation of Au and Zn in terrestrial organisms exposed to Au and ZnO nanoparticles,* American Chemical Society, Preprints of Extended Abstracts 48(1): 334-340, 2008.

[15] Wan, SQ *et al., Effect of Action of Mixture of Two Nano Particles with Two Insecticides to Pest Mite (Epitrimerus pyri)*, Chinese Journal of Pesticides 44(12): 570-572, 2005.

[16] Lin, D *et al., Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth, Environmental Pollution*, Volume 150, Issue 2, November 2007, Pages 243-250.

[17] Lin, D *et al., Phytotoxicity of ZnO nanoparticle: inhibition of ryegrass growth*, American Chemical Society, Preprints of Extended Abstracts 48(1): 276-280, 2008.

[18] Lin, D *et al., Root Uptake and Phytotoxicity of ZnO Nanoparticles*, Environ. Sci. Technol., 2008, 42 (15), pp 5580–5585.

[19] Gamer, AO et al., *Study of the penetration of zinc through dermatomed pig skin in vitro after treatment with a microfine zinc oxide containing formulation (Emulsion mit Z-Cote (Zinkoxid); 10%)*. 2005, BASF Report No. 52H0546/032193

[20] Gamer, AO *et al., The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin*, Toxicol In Vitro. 2006 Apr; 20(3):301-7.

[21] Nohynek, GJ *et al., Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety*, Crit Rev Toxicol. 2007 Mar; 37(3):251-77.

[22] Cross, SE *et al., Human Skin Penetration of Sunscreen Nanoparticles: In-vitro Assessment of a Novel Micronized Zinc Oxide Formulation*, Skin Pharmacol Physiol 2007;20:148-154.

[23] Zvyagin, A *et al., Imaging of zinc oxide nanoparticle penetration in human skin in vitro and in vivo*, J. Biomed. Opt., Vol. 13, 064031 (2008).

[24] Wang, B *et al., Acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice*, Journal of Nanoparticle Research, Volume 10, (2008) 10:263-276.

[25] Sayes, CM *et al., Assessing Toxicity of Fine and Nanoparticles: Comparing In Vitro Measurements to In Vivo Pulmonary Toxicity Profiles*, Toxicological Sciences 2007, 97(1):163-180.

[26] Liu, Zi-hong, *Acute Toxicity of Nano-sized Zinc Oxide in ICR Mice via Intratracheal Instillation*, J Environ Occup Med. Vol. 25 No. 4, 2008.

[27] Ma-Hock, L *et al., Inhalation toxicity of nano-scale zinc oxide in comparison with pigmentary zinc oxide using short-term inhalation test protocol*, Naunyn-Schmiedeberg's Arch Pharmacol (Supplement 1) 2008, 354.

[28] Ma-Hock, L *et al., Report Z-Cote HP1 Subacute 5-day lung toxicity study in male Wistar rats Dust aerosol exposure (DRAFT)*, 2009, BASF Report No. 99I0488/06051.

[29] Conner, MW *et al., Pulmonary damage in guinea pigs caused by inhaled ultra fine zinc oxide, evaluation by light and electron microscopy and analysis of pulmonary lavage fluid*, Microbeam Analysis 21, 589-590, 1986.

[30] Conner, MW *et al., Lung injury in guinea pigs caused by multiple exposures to ultra fine zinc oxide. Changes in pulmonary lavage fluid*, J. Toxicol. Environ. Health 25, 57-69, 1988.

[31] Dinslage-Schlünz, A *et al.*, *The zinc elimination from the rat lung after repeated zinc oxide inhalation*, Beitr. Silikose-Forsch. (Pneumokon) 28, 80-89, 1976.

[32] Lam, HF *et al.*, *Functional and morphologic changes in the lungs of guinea pigs exposed to freshly generated ultra fine zinc oxide*, Toxicol. Appl. Pharmacol. 78, 29-38, 1985.

[33] Lam, HF *et al.*, *Pulmonary function of guinea pigs exposed to freshly generated ultra fine zinc oxide with and without spike concentrations,* Am. Ind. Hyg. Assoc. J. 49, 333-341, 1988.

[34] BASF Report, *REPORT Z-COTE HP1 SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY (DRAFT)*, 2009.

[35] BASF Report, *REPORT Z-COTE MAX SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY (STANDARD PLATE TEST AND PREINCUBATION TEST)*, 2009, Company Study No: 40M0620/064186.

[36] BASF Report, *Z-Cote HP1 MICRONUCLEUS TEST IN BONE MARROW CELLS OF THE MOUSE (DRAFT)*, 2009, Company Study No: 26M0488/064179.

[37] Diembeck, W., *Testing of Zinc Oxide (H&R, PN 104702) with the 3T3 Neutral Red Uptake Phototoxicity Test.*, 2001, Beiersdorf AG Company Report No: 0663-2001.

[38] Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP), *OPINION CONCERNING ZINC OXIDE COLIPA n° S76*, 2003, SCCNFP/0649/03, final.

[39] Dufour, EK *et al.*, *Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese Hamster Ovary cells*, Toxicology Letters, Volume 164, Supplement 1, 20 September 2006, Pages S290-S291.

[40] Cantor, S., *50 humans subject repeat insult patch test skin irritation/sensitisation evaluation*, 1994, AMA laboratories Study Report, Ref no. WPCL 94-141Z/RIPT 00160.SSI.

[41] Kuschner, WG *et al.*, *Pulmonary responses to purified zinc oxide fume*, J Investig Med. 1995 Aug;43(4):371-8.

[42] European Chemicals Bureau, *EU Risk Assessment Report: CAS 1314-13-2*, 2004.

[43] Fink, E., *Repeat photopatch test for photoxicity and photoallergy of HR 96/104702 and HR 96/104702 VeK in healthy adult male and female volunteers*, part 1, 1997, Company Study No: 9066/lib.

[44] Cantor, S *et al., Evaluation of phototoxicity potential by UV-A irradiation on 10 human subjects, 1994, AMA Laboratories Unpublished Report*, Report No: WPCL 94147Z / PHT0016OSSI .10

[45] Jeng, HA *et al., Toxicity of metal oxide nanoparticles in mammalian cells*, J Environ Sci Health A Tox Hazard Subst Environ Eng. 2006;41(12):2699-711.

[46] Mashayekhi, H *et al., Metal Oxide Nanoparticles Show Toxicity to Bacteria*, Preprints of Extended Abstracts Vol 48. No. 1, Proceedings published by the American Chemical Society, 2008.

[47] Brayner R *et al., Toxicological Impact Studies Based on Escherichia coli Bacteria in Ultrafine ZnO Nanoparticles Colloidal Medium*, Nano Lett., 2006, 6 (4), pp 866–870.

[48] Hernandez-Sierra *et al., The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold*, Nanomedicine: Nanotechnology, Biology, and Medicine 4, 237-240.

[49] Jones, N *et al., Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms*, FEMS Microbiology Letters, 2008, Volume 279, Issue 1, Pages 71 – 76.

[50] Zhang, L *et al., Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids)*, Journal of Nanoparticle Research, 2007, 9: 479-489.

[51] Xia, T *et al.; Comparison of the Mechanism of Toxicity of Zinc Oxide and Cerium Oxide Nanoparticles Based on Dissolution and Oxidative Stress Properties*. ACS Nano, 2008, 2 (10), pg 2121–2134.