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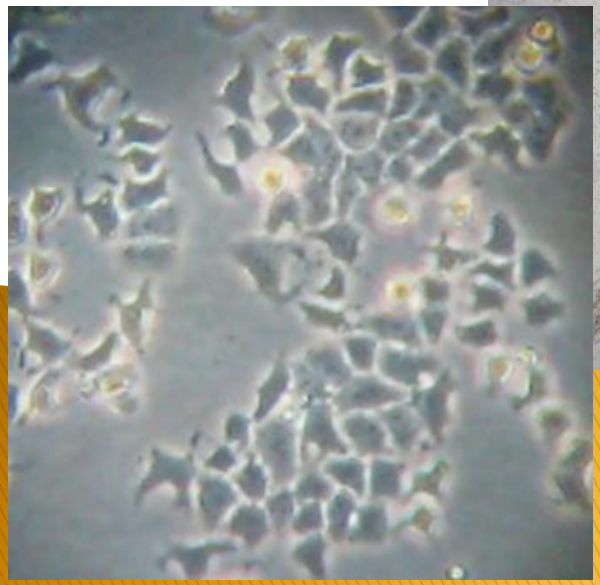
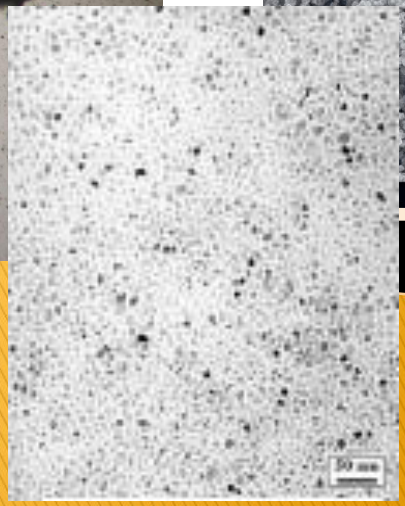
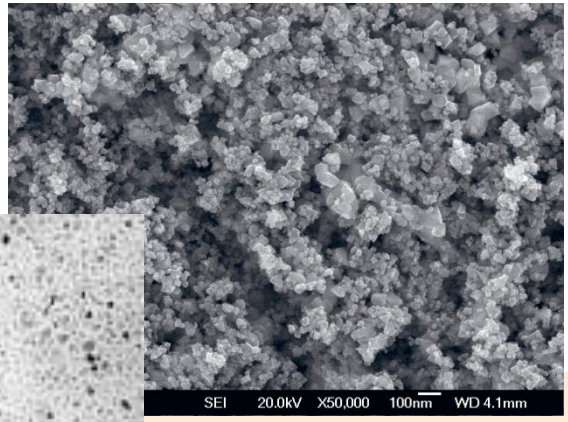
The OECD Sponsorship Program for the Safety Testing of Manufactured Nanomaterials

and the Spanish contribution







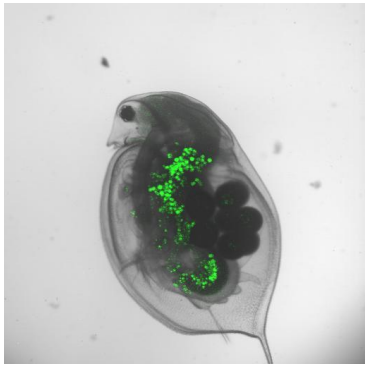
Why assessing the safety of nanomaterials?

- The same characteristics that are making nanoparticles novel and desirable might also produce novel threats to the environment that are different from their parent bulk material.

- Pathways and mechanisms of toxicity are often unknown

- New nanoparticles or modified nanoparticles enter the market at increasing speed.

- According to some sources a thorough testing of all nanoparticles **presently on the market** could take between 34 and 53 years and cost more than 1 billion US$ (Choi et al., 2009).



Problems for assessing toxicity of nanomaterials

- The same nanomaterials can show different behavior depending on the media they are dispersed in

- The same nanomaterials can be different from batch to batch even if they are from the same supplier due to changes in the production process

- Standard protocols for nanoparticle preparation as well as exposure are not harmonized which makes comparisons of experiments difficult

- Environmental or human exposure will seldom happen with pristine particles but rather with particles associated with other substances (e.g. humic acids, constituents of cosmetics…)

- Realistic exposure concentrations are unknown

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The OECD Working party was launched in 2007 and brings together more than 100 experts from governments and other stakeholders from:

a) OECD Countries;

b) non-member economies such as China, Israel, the Russian Federation, Singapore, South Africa, and Thailand; and

c) observers and invited experts from UNITAR, FAO, WHO, ISO, BIAC, TUAC, and environmental NGOs.

Comprises of 9 Steering groups (SGs):

SG1/SG2 OECD database on Manufactured Nanomaterials to Inform and Analyze Human Health and Environmental Safety Research activities

SG3 Testing a representative set of Manufactured Nanomaterials

SG4 Test Guidelines and Manufactured Nanomaterials

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SG5 Co-operation in Voluntary Schemes and Regulatory programs

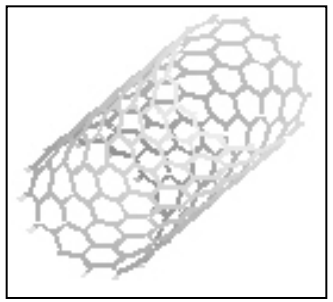
SG6 Co-operation on Risk Assessment

SG7 The Role of *in vitro* Methods in nanotoxicology

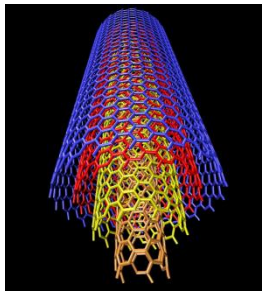
SG8 Co-operation on Exposure Assessment and Exposure Mitigation

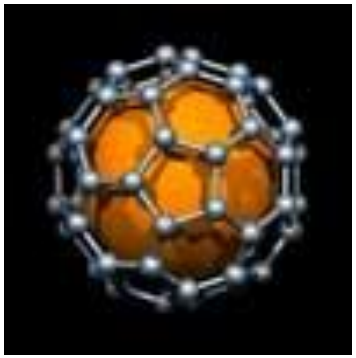
SG9 Co-operation on the Environmentally Sustainable Use of Manufactured Nanomaterials

Nanomaterials tested in SG3



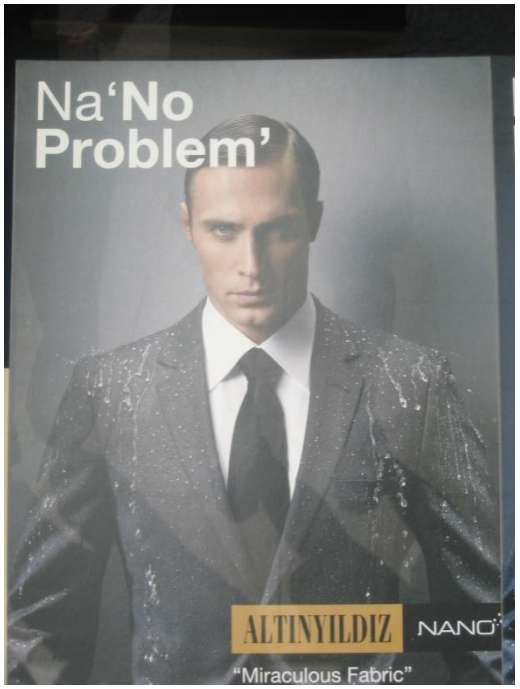
- Fullerenes (C60)
- Single-walled carbon nanotubes (SWCNTs)
- Multi-walled carbon nanotubes (MWCNTs)
- Silver nanoparticles
- Iron nanoparticles
- Titanium dioxide
- Aluminium oxide
- Cerium oxide
- Zinc oxide
- Silicon dioxide
- Dendrimers
- Nanoclays
- Gold nanoparticles





Characteristics of the chosen nanomaterials:

- From a single batch and consistency is guaranteed
- Dispersion protocols have been harmonized as much as possible
- already in production (or close to commercial use)
- Produced in considerable ammounts
- Available for testing
- Information about the material available



Nanomaterial Information/Identification (9 endpoints)
(e.g.) substance name, chemical identity, uses, coating

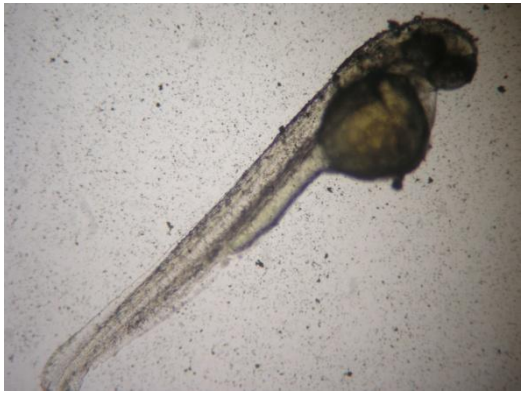
Physical-Chemical Properties and Material Characterization (17 endpoints)
(e.g.) water solubility, particle size, agglomeration/aggregation

Environmental Fate (15 endpoints)
(e.g.) biodegradability, adsorption, accumulation

Environmental Toxicology (6 endpoints)
(e.g.) effects on aquatic and terrestrial organisms

Mammalian Toxicology (9 endpoints)
(e.g.) inhalative toxicity, reproductive toxicity, genotoxicity

Material Safety (3 endpoints)
(e.g.) flammability



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Spain is Co-Sponsor of three nanomaterials:

TiO_2 **CeO_2** **Dendrimers**

And Contributor for one nanomaterial

ZnO

Apart from the OECD WPMN materials, our workgroup at **INIA** also works with:

Gold nanoparticles **Copper nanoparticles** **Graphene**

Employing further assays:

DNA organization (DAP) **Reactive oxygen species (ROS)** **GSH/GSSG**

Glutathion-S- transferase

Various different cell lines

Spain is able to address following endpoints:

Physical-Chemical Properties and Material Characterization *(9 of 17 Endpoints)*

- Agglomeration/ aggregation via TEM, DLS
- Water solubility/ Dispersability via Passive dialysis followed by ICP-MS
- Crystalline phase via TEM, XRD
- Crystallite size via XRD
- Representative Electron Microscopy (TEM) picture(s)
- Particle size distribution – dry and in relevant media via TEM, DLS
- Specific surface area via BET
- Zeta potential (surface charge) via DLS
- Pore size distribution via nitrogen adsorption isotherm

Environmental Fate *(1 of 15 Endpoints)*

- Dispersion stability in water via DLS

Environmental Toxicology *(1 (2) of 6 Enpoints)*

• Effects on pelagic species (short term/long term)

➢ Daphnia acute studies (48h) with mortality as endpoint
➢ Daphnia chronic studies (21d) with fecundity as endpoint
➢Daphnia growth studies (6d) with growth rate as endpoint

➢ Short-term toxicity test on embryo and Sac-fry stages of zebra fish (*Danio rerio*)

• Other relevant information (when available)

Cytotoxicity of nanoparticles to a rat hepatoma (H4IIE) and a trout gonadal (RTG 2) cell line assessed by:

➢ The MTT assay
➢ The NRU assay
➢ The LDH assay
➢ The total protein assay

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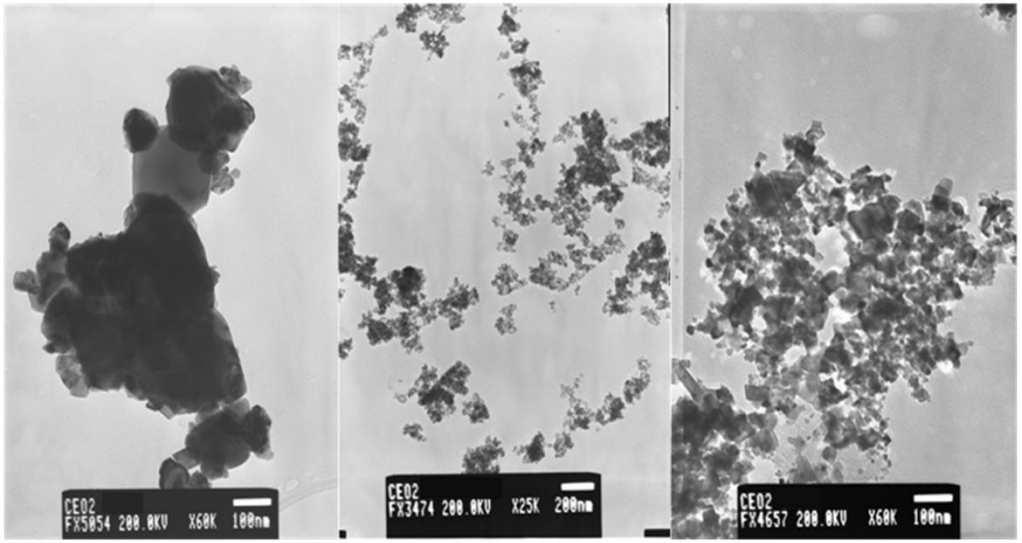
The contribution of Spain to the Sponsorship Programme:

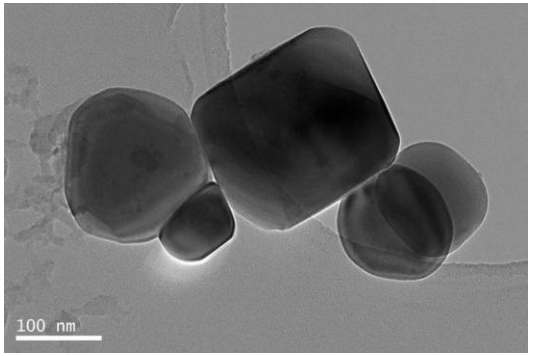
Aim:
To assess toxic effects *in vitro* and *in vivo* with well characterized dispersions of various nanoparticles.

The particles:



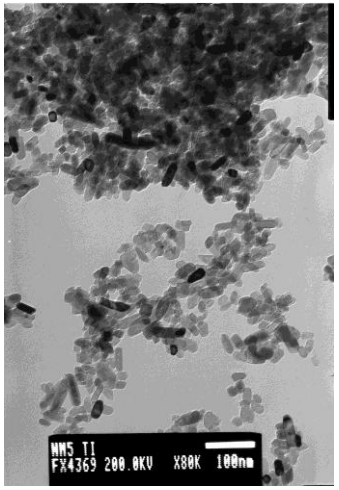
Three CeO_2 particles, a micro sized (left), 10 nm (middle) and 20-25 nm (right)

TiO_2 particles

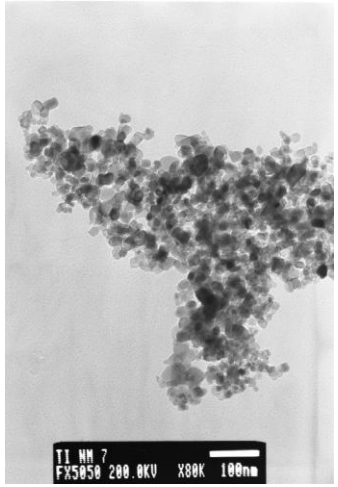


200 nm uncoated

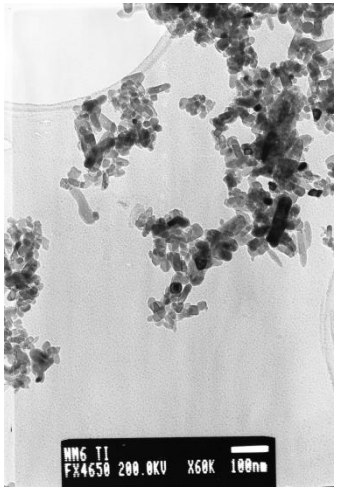
20 nm coated hydrophobic



20 nm uncoated

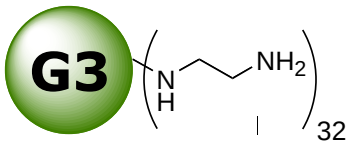


20 nm coated hydrophilic

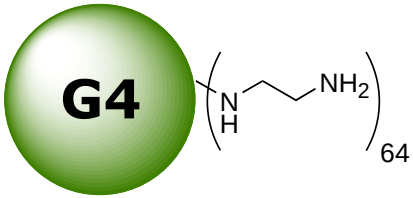


The Dendrimers

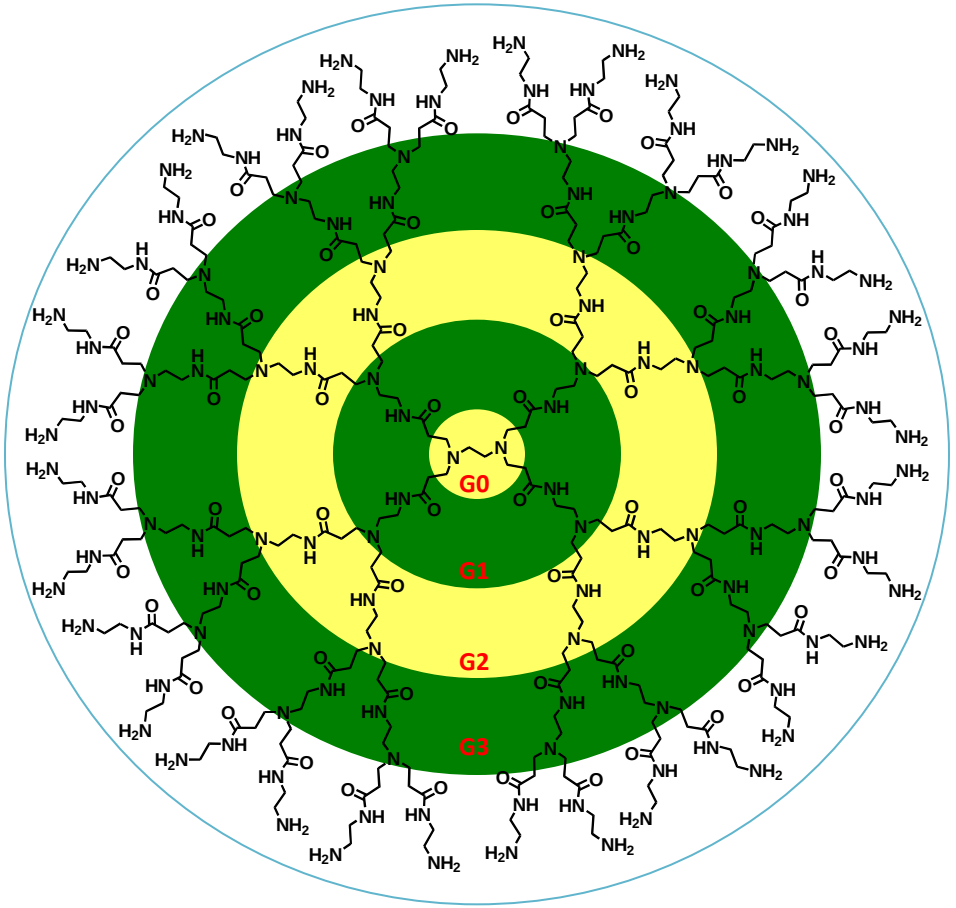
Poly amidoamine (PAMAM) Dendrimers unmodified of the 3rd and 4th generation



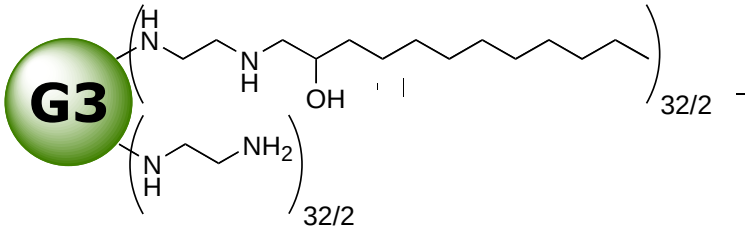
G3-PAMAM-$(NH_2)_{32}$

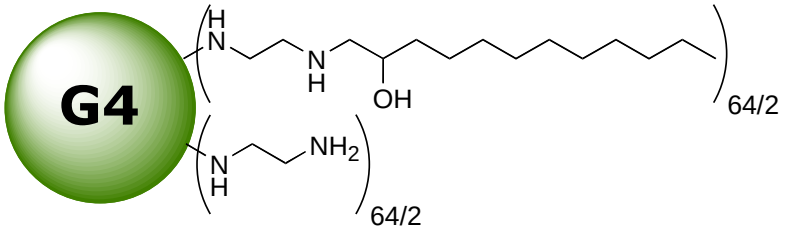


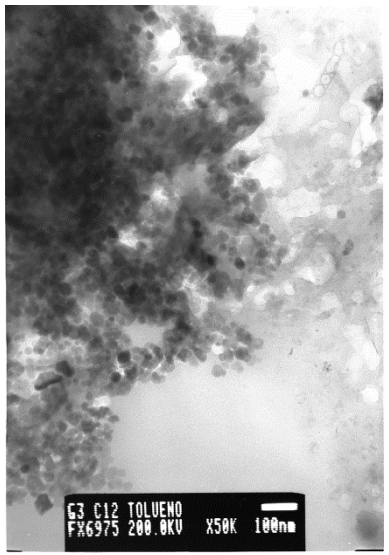
G4-PAMAM-$(NH_2)_{64}$

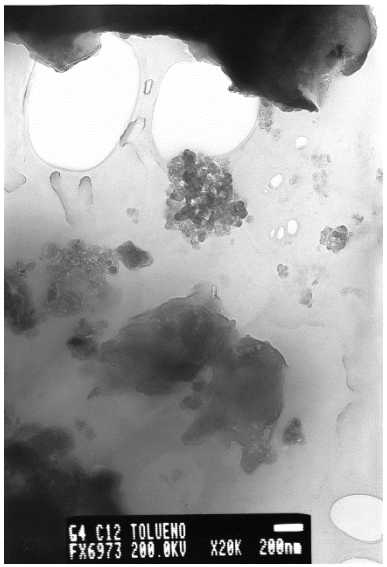


And Dendrimers with 50% of the terminal groups modified









in vitro

4 assays that have one endpoint in common, cytotoxicity, but assess this endpoint through different mechanisms:

The **MTT** assay is based on the ability of living cells to reduce a tetrazolium salt (MTT) to formazan through the **mitochondrial metabolism**

The **neutral red uptake** (NRU) assay is based on the ability of intact lysosomes to retain the dye neutral red in living cells → **lysosomal integrity**

The **lactate dehydrogenase** (LDH) assay asesses the concentration of the cytosolic enzyme lactate dehydrogenase in the cells or the surounding medium. A high concentration of the enzyme in the surounding medium indicates membrane leakage → **membrane integrity**

The **total protein** assay assesses the quantity of proteins in a sample and is an indicator of **cell proliferation**

In vivo

Acute, short term studies are used as range finder studies as well as a hazard estimation. Acute studies are needed to develop a LC_{50}

Chronic, long term studies are used to assess effects of sub lethal concentrations

Both tests are conducted with neonates of *Daphnia magna*, a freshwater filter feeder

Short-term toxicity test on embryo and Sac-fry stages of zebra fish (Danio rerio)

Results for CeO_2 :

Characterization of pristine particles,

Pristine particle size measured by TEM images showed that the two nano sized particles were indeed in the nano range while the size of the micro sized particle was actually just below 100 nm with a considerable standard error

Size distribution measurements of particles in water and media showed aggregates between 200 – 350 nm for concentrations up to 100 μg/mL

Particle suspension stayed stable in water at least over a period of two weeks at a concentration of 500 μg/mL with aggregates between 200 – 400 nm but also > 4 μm

An effect due to nanoparticle exposure can be seen already after 24 h when assessed by the MTT assay. Exposure to micro sized particles has no effect after 24 h for the tested concentrations.

A longer exposure duration, 72 h, showed the observed effects more pronounced, additionally, an effect for the micro sized particle was also visible.

This makes the observed effects **size**, **concentration** and **time dependent**

However:

An assessment of the nominal concentrations used showed that the real concentrations are below the nominal concentrations for all three particles.

Additionally, this effect seems to be particle dependent.

And *in vivo*?

An effect was also visible in a 48 h acute study with *D. magna*, however, only at very high concentrations that might be above environmentally relevant concentrations

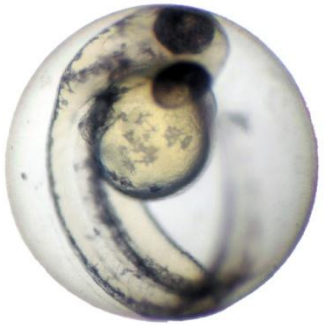
Assessing chronic effects like individual growth rate, fecundity, population growth rate or mean age at first reproduction showed that effects can be observed already at much lower concentrations for the nanoparticles

Control

Oedema

Examples of abnormalities that can be assessed in the zebra fish assay. Elevated occurrences of abnormalities can give hints about toxicity

Abnormal pigmentation

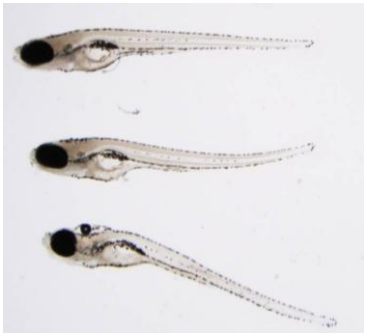


Body abnormality and oedema

Further endpoints:

Length measurement after exposure.

Mortality

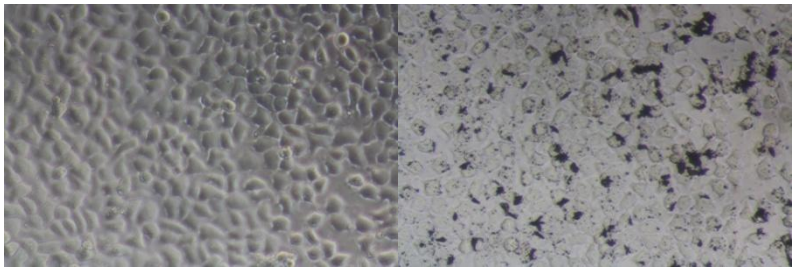


- No effects were observed *in vitro* with other cell lines used or assays employed

- No effects were observed *in vivo* using the zebra fish assay

Conclusions:

- Effect visible size, time and concentration dependent
- More than one assay is needed to reliably assess effects
- More than one test system is needed to reliably assess effects
- Mechanism of toxicity is still unknown
- A thorough characterization is needed to interpret results



Contributers:

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