Ecotoxicity testing for an environmentally safer-by-design nanotechnology industry : an overview

Nanotechnology offers a huge range of opportunities for mankind and the global economy, but needs to be balanced with the assurance that the development of this industry is sustainable with the avoidance of any adverse effects of nanomaterials (NMs) in the environment. Hence the need to study the effects of NMs on organisms in the environment. Although regulation of NMs is still in its infancy, as the appropriate test design systems have not yet be well established, there is a considerable body of ongoing research into the ecotoxicity of NMs and on which the NanoMILE work package 6 is focused.

The environment presents a large, dynamic and highly complex arena. Unlike for human toxicity, where the focus is a single species – man, protection of wildlife needs to consider a very wide range of organism that are representative of the world's ecology. So how and where do you start in considering possible biological effects of NMs in the environment? And, of course, the answer is not a straight forward one. It is clearly not possible to test all organisms and cover all trophic systems. For chemicals, ecotoxicity testing uses a range of representative microbes, plant, invertebrate and vertebrates for terrestrial and aquatic systems. In the world of nanotoxicology the approach is the same, although the test organisms chosen to represent phyla or classes of organisms for protection might differ to best represent the differences in fate and compartmentalisation of NMs in the environment.

In the NanoMILE work package 6, we have chosen a series of organisms representing different phyla, trophic levels and ecological niches, that are considered to be amongst most appropriate for assessing the potential for adverse effects for NMs in the terrestrial and aquatic environments. In selecting suitable organisms as test models, we have factored in many important considerations; including that they have a well-studied biology, to ensure we are best placed to understand an adverse effect, that they have been proven responsive to other environmental contaminants, and that they are relatively easy to maintain in laboratory conditions. The endpoints measured in these test organisms include those indicative of population level effects, for example effects on growth, development and reproduction, as well as a range of biomarkers indicative of particle and/or metal exposure (most of our studies are focused on metal base NMs). In the adopted models collectively WP6 partners consider a very wide range of effect measurements across the different test organisms. In our assessments we are including early life stages, as they are potentially the most vulnerable/sensitive for NM effects, behavioural responses and we are also considering maternal transfer as a route of exposure for NMs to the subsequent generation. We are thus looking to develop a holistic approach in our assessment for possible effects of NMs on the terrestrial and aquatic ecological systems.

Here, the WP6 partners present the organisms they have adopted and the reasons behind their selection as our models to study the ecotoxicology of NMs.

The Terrestrial Environment

The crustacean *Porcellio scaber*.

This is a small, common isopod. It is a member of the crustaceans (along with shrimps, crabs and prawns in aquatic systems). *Porcellio scaber*, is among the most frequently used species in ecotoxicological research. They are becoming popular in nanotoxicological studies as they are small, relatively easily maintained in laboratory cultures and have been widely applied to look at contaminant uptake through whole body measurements. Furthermore, a series of biomarkers have been developed to quantify responses to various contaminants, including metals.

Figure 1: *Porcellio scaber* (Isopoda, Crustacea)

Isopods inhabit the upper layer of the soil and surface leaf litter in a variety of urban and natural habitats. They feed mainly on dead organic material and belong to both meso- and macrofauna and have an average body length between 8 and 12 mm (see figure 1)

These isopods can be dosed easily with NM through their feed. This approach is a natural route of exposure and allows precise control on dosing amount. Feeding rate is a key endpoint studied and this has direct relevance to natural systems, as changes in feeding rates of terrestrial isopods affect the decomposition process and subsequently the matter and energy fluxes through ecosystems.

After isopods ingest the contaminated food, different analyses are conducted, depending on the question that is being addressed. A particular focus in our work has been on the digestive gland (Figure 2).

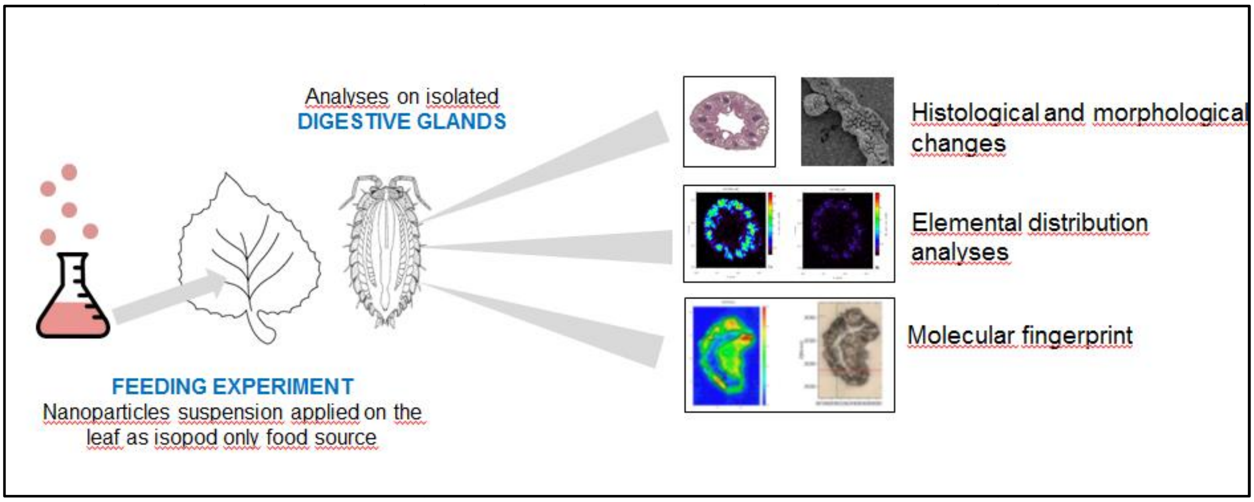

Figure 2: Histological and morphological changes of tissue are observed with light and electron microscopy and with different spectroscopic techniques (infrared spectroscopy). This isopod is particularly useful for studying accumulation of the NMs and their distribution in the different body tissues and this is conducted using elemental distribution analyses with x-ray based techniques.

The worm *Caenorhabditis elegans*

The 1 mm-sized nematode worm *Caenorhabditis elegans* (Figure 3) lives in the solid to liquid phase of soil. It feeds on bacteria that, for their part, consume rotting plant material. Depending on the availability of food, *C. elegans* sustains a boom-bust life style that has made the nematode into one of the super (animal) models due to its ease of cultivation in the laboratory and amenability to genetic and cell biological analyses. *C. elegans* was the first organism that had its whole genome sequenced revealing a notable genetic similarity between worms and humans, especially concerning disease genes. The adult worm lives for 2-3 weeks which allows for a life span-resolved investigation of biological processes including the identification of potential hazards of engineered nanomaterials.

Nanomaterials (NMs) are mixed into the food and there is a very efficient uptake via the intestine into single worm cells. Certain nanomaterials such as silica or silver NMs induce a concentration-dependent acceleration of aging phenotypes in worms such as abated locomotion, neurodegeneration and aberrant amyloid protein clumping. A similar promotion of aging processes has likewise been observed in the worm's reproductive organs and reproductive behavior, respectively.

Currently, we are exploring whether amyloid protein clumping within cells is responsible for the NM-induced premature aging of organ function in *C. elegans*. It is anticipated that neuromuscular processes may represent a major target. Since the nematode possesses a nervous system of 302 neurons that work in a similar manner to humans, we are investigating how elucidating the mechanisms of nanomaterial interactions in this simple animal model, may help to better define potential hazards, environmentally relevant NM-concentrations and cross-species metrics of risk.

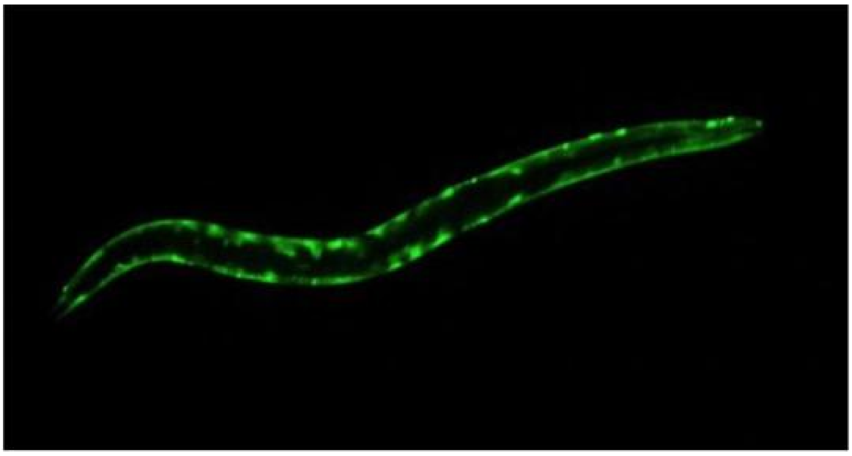

Figure 3: Investigation of neuromuscular fitness in the nematode *C. elegans*. Fluorescent image of an adult reporter worm. The body wall muscle cells in appear as a green fluorescence.

The earthworm (*Eisenia fetida*)

Earthworms (Figure 4) are distributed worldwide in many different soils. They are important members of the soil community due to their ability to change or modify their habitat through their activities. They can improve soil structure, stabilize soil aggregates, increase water infiltration and water-holding capacity as well as increase yield in orchards or grasslands.

Earthworms are substrate feeders; therefore, they are exposed to environmental pollutions over their inner body surface but also over their outer body surface via direct contact with the soil pore water. Although several species of earthworms have been used in ecotoxicological testing, only the two closely related species *Eisenia fetida* and *Eisenia andrei* have been included in standardized testing guidelines. Their susceptibility to chemicals is considered to be representative of most soil earthworm species and the effects of chemicals on them are relatively well known, due to required tests with these animals for the registration of plant protection products for more than three decades.

Figure 4: Earthworms (*Eisenia fetida*)

Earthworms have been key in assessing both pristine and naturally aged NMs that have undergone transformation within the soil. In our work to assess long term effects of silver NMs, we have shown that the chemical make-up of the soil significantly dictated toxicology responses in the test species. This increases the awareness that standardised test guidelines for certain species may need to consider more ecologically relevant experimental conditions to achieve meaningful results with regard to environmental risk assessment.

The Aquatic Environment

The microalga *Chlamydomonas reinhardtii*

When attempting to represent a high variety of wildlife taxa it would be remiss to overlook aquatic systems which are sinks for accumulating toxicants. Primary producers, such as microalgae, comprise the base of the food chain from which effects can be transmitted to higher trophic levels. Therefore, the estimation of bioaccumulation and toxicity to primary producers are important for accurate risk assessment and *C. reinhardtii* is an excellent model in this regard.

C. reinhardtii is a single celled green algae which is commonly found in freshwater. The alga is a species that reproduces sexually or asexually and is about 10 µm in diameter with a glycoprotein rich cell wall, a large single chloroplast, a nucleus, an eyespot which senses light, a pyrenoid which stores starch and two anterior flagella for motility. *C. reinhardtii* can be cultured easily in the lab with a short generation time of 8 to 12 h and is amenable to genetic manipulation, with a vast array of functional mutants available. It has three genomes: the nuclear, the mitochondrial and the chloroplastic, all of which have been sequenced and fairly well annotated.

C. reinhardtii is routinely used in ecotoxicological risk assessment as one of the standard organisms for testing effects of toxicants in fresh water. The routine tests mostly focus on the inhibition of growth which represents an adverse effect at the population level and requires at least 24 hours. A more detailed assessment of different physiological endpoints is used to study the mechanisms of toxicity. One such physiological endpoint is the inhibition of photosynthetic yield, which can be quantified already within minutes after exposure. Another is ATP content, an indicator of the viability and physiological state (e.g., stress) of the algae.

The majority of studies for nanotoxicology deal with freshly prepared NM suspensions. However, during their life-cycle nanoparticles undergo modifications in the environment, which can play an important role on the toxicity and this is a major focus of work on this organism within NanoMILE. In a model waste water treatment plant, most silver tends to occur in a sulphadised form in the sludge and in the effluent. The fate and toxicity of the chemically transformed nanoparticles in the freshwater system (aged NMs) differ from the freshly prepared suspension and is one of the challenges for understanding the toxicity in environmentally realistic conditions.

Figure 5: The microalga *Chlamydomonas reinhardtii*

The zebrafish (*Danio rerio*)

The zebrafish is one of the most widely adopted fish models in ecotoxicology. The zebrafish has been used as an in vivo model organism in a wide range of fields across biological and medical sciences, and the associated biological understanding, genome resources and proven track record as a robust laboratory model, makes it an ideal for nanotoxicity assessments. The advantages of high fecundity, embryo transparency, fast and well-characterized development, gene manipulation accessibility, short reproduction time, responsive to short-term acute toxicity assays as well as long-term exposure experiments facilitate a wide range of assessment types. The zebrafish offers higher-order biological processes and multi-organ responses that can be incorporated into ecologically representative exposure method for environmental relevance. The use of zebrafish embryos in tests on NMs facilitates high throughput[1] in a vertebrate organism.

Figure 6: Zebrafish

The live-bearing fish *Xenotoca eiseni*

One concern for NMs in humans is their potential to cross the placenta and accumulate in the developing foetus at a time when the development is most vulnerable to the effects of toxicants. In NanoMILE we are investigating the use of a new model to study maternal transfer of NM, *Xenotoca eiseni. X. eiseni* is a live-bearing (viviparous) fish and has morphological and functional adaptations in the female and embryo to facilitate the transfer of nutrients and oxygen from the maternal organism to the embryo. The species has developed a specific exchange organ, akin to an ancient placenta system as seen in mammals. This matrotrophic pathway provides a fantastic system for studying the uptake of NMs across a placental style pathway. In NanoMILE we are dosing NMs into the diet of adult *X.eiseni* and measuring NM uptake into the developing young and investigating for any biological effects. This work is focused on silver NMs.

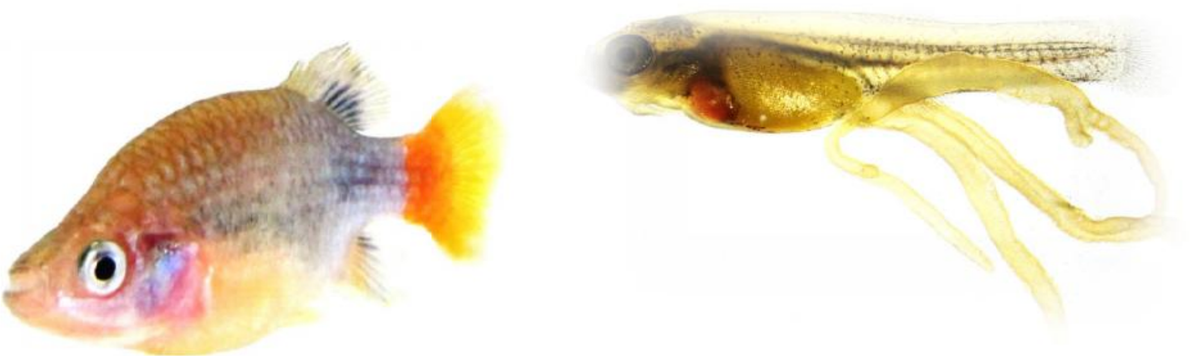

Figure 7: X. eiseni (right) and Trophotaeniae of larval X.eiseni 4 weeks post fertilisation. These prominent structures absorb maternally derived nutrients from a surrounding fluid matrix

[1] High-throughput screening (HTS) is a method of automated testing, allowing a high number of tests simultaneously (e.g. one cell type in a solution with different compounds at different concentrations).