

An integrated multispecies two-tiered approach for the environmental risk assessment of nanomaterials: a case study with Ag NPs

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Due to the great increase in the use of nanomaterials for a variety of biomedical, domestic and industrial applications, the input of nanoparticles (NPs) and other nanomaterials in the aquatic environment is expected to rise in the following years. In spite of this, there is only limited information on the fate, distribution and toxicity of NPs to aquatic organisms. The aims of our study were to investigate the bioavailability of metal bearing NPs in mussels *Mytilus galloprovincialis* and zebrafish *Danio rerio* and to determine the possible adverse effects of NPs in comparison to bulk and ionic forms on the same target organisms. For this, a two-tiered strategy was developed combining both *in vitro* and *in vivo* approaches. *In vitro* techniques provide a quick and reproducible tool for the screening of NP toxicity. In tier 1, cytotoxicity of a variety of NPs (Au, ZnO, SiO₂, TiO₂, CdS, Ag and CuO) at a wide range of concentrations was tested in isolated mussel hemocytes and gill cells and LC50s were calculated. In tier 2, sublethal concentrations below the LC25 were selected to investigate *in vitro* uptake and reactivity of NPs and to discover putative mechanisms of toxicity in both cell types. *In vivo* studies with mussels comprised short-term (1-3 d) experiments to determine bioavailability and lysosomal membrane stability as a general indicator of health (tier 1) and medium-term (21 d) experiments to assess adverse effects using a battery of molecular, cellular and tissue-level biomarkers (tier 2). Similarly, studies in zebrafish started with short-term embryo toxicity tests (tier 1) followed by 21 d experiments (tier 2) in case significant toxicity was found in tier 1. Results allowed to classify studied NPs based on their toxicity to mussel cells *in vitro* and to zebrafish embryos. Overall, NP toxicity depended on their physico-chemical properties and their behaviour in exposure media. Maltose-coated Ag NPs of 20 nm resulted the most toxic NPs tested in the two model organisms. At sublethal concentrations, Ag NPs increased ROS production and induced antioxidant enzyme activity, increased DNA damage and activated lysosomal acid phosphatase activity and multixenobiotic resistance MXR transport activity in mussel cells. Further, Ag NPs decreased Na-K-ATPase activity in gill cells and affected the actin cytoskeleton in hemocytes. Exposure to the ionic form of Ag produced similar effects, although at a higher magnitude, suggesting that observed responses were due at least in part to dissolved Ag. Exceptionally, the stimulatory effect on hemocyte phagocytic activity was nanoparticle specific. In agreement, in *in vivo* experiments with mussels exposed to the same NPs, Ag was accumulated significantly in soft tissues, being localized mainly in the endo-lysosomal system at the subcellular level. This was associated to a significant reduction in lysosomal membrane stability and altered the structure of the digestive gland as a result of massive digestive cell loss. Accordingly, Ag NPs were highly toxic to developing zebrafish embryos, causing mortality or a variety of severe malformations at lower exposure concentrations. Exposure of adult zebrafish to the same Ag NPs resulted in Ag internalization that lead to an array of sublethal effects from changes in liver transcriptome to gill histopathologies. Obtained data may contribute to the risk assessment of nanomaterials in the aquatic environment.

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