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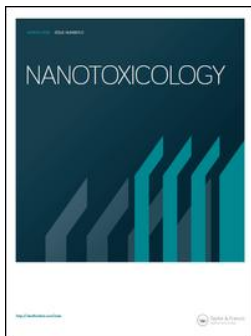
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ORIGINAL ARTICLE

# Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles

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## Abstract

Regulatory ecotoxicity testing of chemicals is of societal importance and a large effort is undertaken at the OECD to ensure that OECD test guidelines (TGs) for nanomaterials (NMs) are available. Significant progress to support the adaptation of selected TGs to NMs was achieved in the context of the project MARINA (<http://www.marina-fp7.eu/>) funded within the 7th European Framework Program. Eight OECD TGs were adapted based on the testing of at least one ion-releasing NM (Ag) and two inert NMs (TiO<sub>2</sub>). With the materials applied, two main variants of NMs (ion releasing vs. inert NMs) were addressed. As the modifications of the test guidelines refer to general test topics (e.g. test duration or measuring principle), we assume that the described approaches and modifications will be suitable for the testing of further NMs with other chemical compositions. Firm proposals for modification of protocols with scientific justification(s) are presented for the following tests: growth inhibition using the green algae *Raphidocelis subcapitata* (formerly: *Pseudokirchneriella subcapitata*; TG 201), acute toxicity with the crustacean *Daphnia magna* (TG 202), development toxicity with the fish *Danio rerio* (TG 210), reproduction of the sediment-living worm *Lumbriculus variegatus* (TG 225), activity of soil microflora (TGs 216, 217), and reproduction of the invertebrates (*Enchytraeus crypticus*, *Eisenia fetida*, TGs 220, 222). Additionally, test descriptions for two further test systems (root elongation of plants in hydroponic culture; test on fish cells) are presented. Ecotoxicological data obtained with the modified test guidelines for TiO<sub>2</sub> NMs and Ag NM and detailed method descriptions are available.

## Keywords

Environment, engineered nanomaterials, hazard assessment, nanoparticles, OECD test guidelines, regulatory toxicology, silver, titanium dioxide, test validation

## History

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## Introduction

The standardization of regulatory toxicity tests is central to EU wide confidence in hazard and risk assessment in order to identify adverse human health and environmental effects of new substances, including nanomaterials (NMs). The Organization for Economic Cooperation and Development (OECD) set up a Working Party on Manufactured Nanomaterials (WPMN) in 2006. One of the aims of the WPMN is to enhance the understanding of the applicability of the OECD test guidelines (TGs), designed for chemicals, to NMs. The results from OECD TGs are intended to be accepted by all EU member states by the

OECD agreement on Mutual Acceptance of Data in the Assessment of Chemicals (the MAD principle, <http://www.oecd.org/env/ehs/mutualacceptanceofdatamad.htm>). However, the MAD principle requires confidence in the test protocols for all new chemical substances, and some difficulties in conducting the TGs with NMs have been reported.

In 2009, the WPMN evaluated the applicability of the OECD TGs, which were originally developed for traditional chemicals, to NMs (OECD, 2009). With a focus on the ecotoxicological test guidelines, the conclusion was that in principle, the TGs are considered to be suitable also for the testing of NMs; although some adaptations were found to be necessary. Guidance was then developed on sample preparation and dosimetry for the safety testing of manufactured NMs, which also encompassed these issues for ecotoxicological testing (OECD, 2012). However, this document only provided general guidance on the broad issues related to sample preparation and dosimetry (OECD, 2012). It did not give, and was not intended to provide, specific guidance on the details of individual toxicity tests. Hence, a more detailed description was required. In 2013, an OECD “WPMN Expert Meeting on Environmental Fate & Eco-Toxicology” was held to further evolve the environmental testing of NMs. Several topics

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and test guidelines were comprehensively discussed (Kühnel & Nickel, 2014; OECD, 2014). Another workshop was organized in Washington in July 2014 focused on drafting an OECD guidance document on aquatic and sediment toxicology testing of nanomaterials. This is related to an OECD project approved in 2013 and aimed to provide the necessary amendments to existing OECD aquatic toxicity test methods. A paper summarizing the results of the workshop and the agreed recommendations from experts has been recently published (Petersen et al., 2015). Nevertheless, currently no compilation of proposed nano-specific test modifications on OECD TGs for ecotoxicity is available and therefore, the adaption of the test guidelines is hampered.

In this context, significant progress on providing experimental data to support proposals for the adaptation of several OECD TGs was achieved by the project MARINA (<http://www.marina-fp7.eu/>), funded within the 7th European Framework Program (FP7). The aim here is to provide an overview of the progress on ecotoxicity testing protocols with a focus on the information requested by regulatory bodies for safety assessment of NMs. This compilation presents a summary of the difficulties identified with the OECD TGs for NMs, the proposed modifications and where to find further in-depth information. Eight OECD test guidelines were addressed including: growth inhibition using the green algae *Raphidocelis subcapitata* (formerly: *Pseudokirchneriella subcapitata*; TG 201), acute toxicity with the crustacean *Daphnia magna* (TG 202), development toxicity with the fish *Danio rerio* (TG 210), reproduction of the sediment-living worm *Lumbriculus variegatus* (TG 225), activity of soil microflora (TGs 216, 217), and reproduction of the invertebrates (*Enchytraeus crypticus* and *Eisenia fetida*, TGs 220, 222). The scientific and practical modifications we propose are briefly justified, and rather than give a detailed account of the justification here, for the sake of clarity in this overview, the available background information is provided by referring to already published articles on individual test systems or specific subjects.

Additionally, tests on the root elongation of terrestrial plants in hydroponic systems (Liu et al., 2016) and on fish cells were performed. Procedures for these tests were developed as part of the MARINA project as no OECD TGs were available. The test on fish cells was performed as an *in-vitro* assay to explore its feasibility for obtaining information for the ranking and grouping of NMs and as a potential alternative for fish *in-vivo* testing. The testing of plants in hydroponic systems serves as the intermediate step of testing a terrestrial species. As proof of principle, ecotoxicity testing results of three selected NMs (silver: NM-300K and TiO<sub>2</sub>-nanomaterials: NM-104, NM-105 from the JRC Nanomaterials Repository) investigated in these test systems are summarized as Supplemental information (S4).

## Methods

The OECD TGs addressed are listed in Table 1. The details of the NMs used for the investigations are included as Supplemental

information (S1). Comprehensive descriptions of the applied test systems, specifically outlining the modifications and adaptations proposed for nanomaterials are added as Supplemental information (S2).

The test on fish cells addressing mitochondrial activity, plasma membrane integrity and lysosome functionality as endpoints was performed as an *in-vitro* assay. The testing of plants was performed hydroponically to enable proper control of the fate of the NMs being tested. Detailed descriptions of these additional methods are also included in the Supplemental information (S3).

## Results

Based on the investigations and results obtained with various NMs, an overview of the modified OECD test procedures with their justification for NMs and references for in-depth information is presented. For every test system the protocol was developed individually as the demands of the various test organisms differ. A common difficulty in aquatic tests was the sedimentation of NMs in the test media. Different approaches have to be applied to avoid sedimentation and to achieve the intended exposure concentrations for the duration of the tests. This is essential to achieve maximum contact between test organism and the NMs. In the test with algae, the vessels are shaken during the incubation period so that the NMs and algae are continuously in close contact. No further measures were considered to be necessary. However, EDTA as a component of the test medium can interfere with metallic NMs causing Fe precipitation resulting in unfavorable growth conditions. Therefore, modifications of the test medium can improve the results. In the fish test, sedimentation of the NMs can be reduced by stirring to generate turbulence. But a modified incubation system is also needed to protect the larvae from mechanical injury and to further improve the exposure of the fish. A modification of the test medium (water) for fish does not seem to be necessary. In contrast to algae and fish, daphnids are very sensitive towards turbulence. Shaking or stirring is not an option to keep NMs in dispersion for this delicate organism. Instead, adapting the composition of the test media seems to be the method of choice.

The data set obtained with the modified methodological approaches is available as Supplemental information (S4). With regard to the test conduct, the recommended adaptations of the test guidelines are listed as follows:

### OECD TG 201: freshwater alga and cyanobacteria, growth inhibition test

*Topic:* Interference of NMs with components of test medium resulting in negative impact on growth.

*Proposed modification:* The chelating agent EDTA can interfere with metal NMs and a modified version of OECD algal medium (OECD-M) for *Raphidocelis subcapitata* is

Table 1. OECD test guidelines for ecotoxicological testing whose adaptation to nanomaterials was investigated in the European FP7 project MARINA (<http://www.marina-fp7.eu/>).

OECD TG <sup>a</sup>	Medium	Test organism	Test species used in the project	Endpoint
201	Water	Algae	<i>Raphidocelis subcapitata</i>	Growth rate
202	Water	Daphnids	<i>Daphnia magna</i>	Immobilization
210	Water	Fish	<i>Danio rerio</i>	Survival, hatching, morphometrics
216	Soil	Terrestrial Microorganisms	Soil microflora	Nitrogen transformation
217				Carbon transformation
220	Soil	Worms	<i>Enchytraeus crypticus</i>	Reproduction
222	Soil		<i>Eisenia fetida</i>	Reproduction
225	Sediment	Sediment worms	<i>Lumbriculus variegatus</i>	Emergence

<sup>a</sup>Number of OECD test guideline.



proposed. This EDTA-free algal medium should be considered when testing metal nanomaterials.

*Justification:* With the modified medium, it is possible to omit the chelating agent EDTA, which may be advantageous when investigating the toxicity of metal NMs, particularly in the presence of natural organic material.

Another major modification of OECD medium, which permits good growth is that it has iron supplied as FeSO<sub>4</sub> rather than FeCl<sub>3</sub>. The amount of phosphorus supplied has also been increased 5× and was supplied as equimolar monobasic and dibasic salts.

For further details see Supplemental information S2.

*Topic:* Interference of NMs with the determination of the biomass by cell counting.

*Proposed modification:* Measurement of biomass by determination of *in-vitro* Chlorophyll A (Chla).

*Justification:* All existing measures of algal biomass have disadvantages when used to test particulate materials.

TiO<sub>2</sub> interferes with optical density (OD) readings in a non-linear fashion. It also interferes very strongly with *in-vivo* fluorescence. We have also found that *in-vivo* fluorescence can be an unstable parameter, depending on the prior light exposure of the culture: repeated measurement of fluorescence on the same sample yields different results. These considerations apply also to other NMs tested in concentrations high enough leading to possible measurement artifacts.

Cell counting in a hemocytometer is very laborious and has a large variance. It also may not truly reflect biomass if the mean cell size changes in response to a toxicant or other conditions. Determination of biomass by fluorometry following Chla extraction avoids the interference that TiO<sub>2</sub> and other particles cause when measuring optical density and *in-vivo* fluorescence.

Measuring *in-vitro* chlorophyll may not accurately reflect biomass if the chlorophyll content of cells changes because of toxicants or changes to the light regime. Nevertheless, we believe this is the most reliable practical measure of biomass under these conditions.

#### OECD TG 202: acute immobilization of *Daphnia magna*

*Topic:* Sedimentation of NMs and reduced exposure concentrations for daphnids.

*Proposed modification:* Testing at pH-values where more stable dispersions are obtained and using a very low ionic strength medium with low amounts of divalent cations (e.g. Very Soft EPA medium) under which *D. magna* can grow and reproduce normally.

*Justification:* Daphnids are sensitive towards mechanical turbulence. Modified test media are an alternative to increase the stability of NMs in dispersion and the exposure concentrations for daphnids. Based on information on zeta potential measurements and point of zero charge, a pH range and media composition can be identified, where each NM suspension have low agglomerate hydrodynamic diameters and can be kept better suspended during test incubation.

*Reference:* Cupi et al. (2015); Cupi et al. (2016).

#### OECD TG 210: fish, early-life stage toxicity test (with zebrafish)

*Topic:* Sedimentation of NMs and reduced exposure concentrations for fish.

*Proposed modification:* Use of exposure chambers coupled with water changes every 24 hours in order to improve NM dispersion during tests. A prototype exposure chamber is described in Boyle et al. (2015), and our additional modifications include a filter mesh to protect the fish embryos from the risk of mechanical injury and mixing of the water without an air lift.

*Justification:* In the current experiment, the test materials settled out of the water column and it was therefore difficult to maintain test concentrations of NM within ±20% of the intended nominal concentration (as required in the OECD 210 TG). For the same reason it was also not possible to maintain test concentrations above the 80% limit often required in regulatory toxicity.

*Reference:* Boyle et al. (2015); For further details of the exposure chamber see also Supplemental information S2.

*Topic:* Sensitive life-stages of fish for the testing of NMs.

*Proposed modification:* Conduct tests on hatched larvae only.

*Justification:* It was seen that larvae were more sensitive than embryos to the NMs tested here. This notion is corroborated by several previous studies which generally show that while NMs are sometimes toxic to unhatched embryos, the hatch larvae show greater toxicity and evidence of internal uptake of the test materials (review, Handy et al., 2011). Whilst the use of embryos in toxicity testing is encouraged as an alternative to free living vertebrate animals, this should not be at the risk of increased uncertainty in hazard and risk assessment (Oris et al., 2012).

*Reference:* Handy et al. (2011).

*Topic:* Criteria and methodologies for the assessment of sublethal effects.

*Proposed modification:* The TG should include more clearly defined criteria and methodologies for the assessment of sublethal effects.

*Justification:* This section of the original OECD 210 TG lacked clear definition and direction and also failed to take into account the characteristics of different fish species. All the traditional endpoints (e.g. fish body length, muscle block width, yolk sac volume and dimensions of the head of the larvae) used in sub-lethal investigations seem to be useful to NMs and it is the magnitude to the response that alters with NM exposure. Although the re-issued 210 TG in 2013 provided more information on how to measure fish length, the methodology for morphometrics and other sublethal endpoints are not sufficiently described for work with NMs.

*Reference:* Shaw et al. (2016).

#### OECD TG 216: nitrogen transformation test

*Topic:* Natural soils suitable for the detection of effects.

*Proposed modification:* For the testing of ion releasing metal NMs, the pH of the soils should be at the lower end of the range accepted according to the test guideline (pH 5.5).

*Justification:* Effects of these metallic nanomaterials are more pronounced at lower pH. A pH of 7 is often achieved by the addition of lime and untreated soils usually have a lower pH. A pH of 5.5 is considered as a compromise of increased safety and environmental relevance.

*Reference:* Schlich and Hund-Rinke (2015).

*Topic:* Homogeneity of spiking.

*Proposed modification:* NMs should be added to each replicate, except for low concentrations.

*Justification:* The homogenous mixing of NMs in the soil can be difficult. Hence, it is proposed to mix the NMs in the individual replicates to ensure the same content and a homogenous mixture exists in each replicate. This may be difficult for low concentration, hence in these it is recommended to mix enough soil and NMs for all replicates together and then divide the mixture into aliquots.

*Topic:* Detection of effects due to slow transformation of NMs in soil.

*Proposed modification:* Extension of test duration to 56 days.

*Justification:* Various nanomaterials show effects after aging. Release of ions is a kinetic process. Delayed effects were already observed for several CeO<sub>2</sub> nanomaterials (not published). It is assumed that a modification of the crystalline structure is the reason. The proposed extension of the test period to 56 days is a compromise of increased safety and practicability.

*Reference:* Schlich and Hund-Rinke (2015); Schlich et al. (2013).

*Topic:* Nitrogen source suitable for the detection of effects of NMs.

*Proposed modification:* Instead of a complex organic nitrogen source such as Lucerne meal, an inorganic nitrogen source (e.g. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) is recommended for the testing of nanoparticulate metals or metal oxides.

*Justification:* Released ions sorb to the complex organic nitrogen source and their bioavailability is reduced. Usually, the transformation of ammonium to nitrate is very sensitive. The application of an inorganic nitrogen source results again in a sensitive test system, which can be used as an early warning system, although in the environment organic material may be present. This can be considered in the scope of a hazard refinement.

*Reference:* Hund-Rinke and Schlich (2014).

*Topic:* Sensitive detection approach regarding nitrification.

*Proposed modification:* Multiple short-term determinations of the potential ammonium oxidation activity (ISO Guideline 15685, 2012) during the incubation period instead of the determination of the long-term nitrification process from bound nitrogen to nitrate.

*Justification:* According to the test guideline OECD 216, a nitrogen source is added at the start of the incubation period and the formation of nitrate is measured. For non-agrochemicals, nitrate is measured at the start and end of the test. Recovery after an initial effect of NMs on the nitrifiers cannot be detected by this procedure. Determination of the potential ammonium oxidation activity eliminates this problem. The nitrogen source is added at the time of the measurement and not at the start of the incubation period. Additionally, the incubation period of the treated soil can be easily extended. The investigation period is not limited by a complete transformation of the nitrogen source added at test start. With the potential ammonium oxidation activity at least one step of the nitrification is covered (transformation of ammonium to nitrite). This approach has been found to be a very sensitive toxicity indicator.

*Reference:* Hund-Rinke and Schlich (2014).

### OECD TG 217: carbon transformation test

*Topic:* Soils suitable for the detection of effects.

*Proposed modification:* For the testing of ion releasing metal NMs, the pH of the soils should be at the lower end of the range accepted according to the test guideline (pH 5.5).

*Justification:* see OECD 216 "Nitrogen transformation test".

*Reference:* Schlich and Hund-Rinke (2015).

*Topic:* Homogeneity of spiking.

*Proposed modification:* NMs should be added to each replicate, except for low concentrations.

*Justification:* see OECD 216 "Nitrogen transformation test".

*Topic:* Detection of effects due to slow transformation of NMs in soil.

*Proposed modification:* Extension of test duration to 56 days.

*Justification:* see OECD 216 "Nitrogen transformation test".

*Reference:* Schlich and Hund-Rinke (2015); Schlich et al. (2013).

### OECD TG 220: enchytraeid reproduction test, OECD TG 222: earthworm reproduction test

*Topic:* Artificial soil reduces the bioavailability of NMs due to sorption to soil organic matter.

*Proposed modification:* For tests in artificial soil, it is recommended to reduce the organic matter content of the standard OECD test soil from 10 to 5 or 2%.

*Justification:* By the reduction of organic matter a larger area of agricultural conditions are reflected, potential reference type soil candidates with 2% organic matter are already available. In testing, it has been shown that soil with higher organic matter has greater EC<sub>x</sub> values, i.e. there is a lower toxicity.

*Topic:* Natural soils.

*Proposed modification:* Comparable to soil microflora, the soil pH can be modified where relevant for ion releasing metal NMs.

*Justification:* see OECD 216 "Nitrogen transformation test."

*Topic:* Homogeneity of spiking.

*Proposed modification:* NMs should be added to each replicate, except for low concentrations.

*Justification:* see OECD 216 "Nitrogen transformation test."

*Topic:* For enchytraeids: detection of effects due to slow transformation of NMs in soil.

*Proposed modification:* Extension of test duration to 42 days and measurement of more life stage endpoints, i.e. hatchability, juvenile growth and time to maturity.

*Justification:* In contrast to the earthworm test with test duration of 56 days, the incubation period of enchytraeids is 4 weeks. The test should be extended due to transformation of NMs or possible slower uptake and different toxicity during the various life stages (see also test with microflora: OECD 216, OECD 217).

*Reference:* Bicho et al. (2015).

### OECD TG 225: sediment-water lumbriculus toxicity test using spiked sediment

*Topic:* Reduced bioavailability of NMs due to sorption to organic matter.

*Proposed modification:* Lower organic matter sediment (2% peat as opposed to 5%).

*Justification:* The modification makes the sediment in line with the recommendation described in OECD TG 315 (OECD TG 315, 2008) which recommends 2% peat content in order to correspond to the organic matter content of many natural sediments. Bioavailability of the NMs is increased by the decreased peat content and determined effects are considered to better reflect the environmental behavior. Worms still displayed normal behavior and reproduced well in control sediments.

*Topic:* Homogeneity of spiking.

*Proposed modification:* Applying NMs directly to each sediment replicate.

*Justification:* OECD 225 recommends spiking an isolated, dry sand portion of sediment, which is subsequently dried and incorporated into the remainder of the sediment. Direct application (or spraying) of NMs to sediments could be an idea for a potentially more environmental realistic application method (Handy et al., 2012). Direct application potentially limits variation in NM concentrations in comparison to bulk spiking, whilst limiting opportunities for NM to adhere to mixing equipment and additional glassware. Also, from a practical point of view, direct application is a more time efficient method, especially when multiple concentrations are to

be tested simultaneously. However, to prove the usefulness the comparability to the current recommendation in the OECD TG still has to be shown.

No specific instructions are given regarding sediment mixing, thus an industrial food mixer was initially used for the mixing of sediment components. As sediments were spiked individually, beakers were placed on a shaker and left overnight to achieve a homogenous mixture of NMs.

## Discussion

Chemicals legislation is intended to protect the environment from the risks that can be posed by chemicals. For regulatory purposes, highly standardized procedures have to be applied to ensure the comparability of results. Laboratory tests as described in the OECD test guidelines on ecotoxicity intend to evaluate, select and rank chemicals. Many test approaches and variations in the testing of NMs are described in the literature to provide information on the effect of different parameters on the ecotoxicity of NMs, but currently no agreed guidance is available.

For a robust revision of testing methods for NMs several subjects have to be considered. An essential step in ecotoxicological testing is the spiking of the test media with NMs. Techniques and protocols for dispersing nanomaterials are an important issue and available information on protocols for the preparation of stock suspensions was summarized by Hartmann et al. (2015). Methods for spiking solid test media are also available (Hund-Rinke and Klawonn, 2013; Hund-Rinke et al., 2012; Scott-Fordsmann et al., 2008). A comprehensive characterization of the NMs is necessary to interpret the results. This topic is also addressed in various papers (Laborda et al., 2016; Montañó et al., 2014; OECD, 2012) and should be performed using the latest techniques, and at least following the recommendations in the OECD WPMN document (OECD, 2012). With the present compilation of recommended modifications to ecotoxicological test guidelines, elaborated in the scope of the EU-project MARINA, the further standardization of test guidelines for the testing of NMs shall be supported. The information on modifications is also of relevance in the context of general guidance documents such as the guidance on sample preparation and dosimetry (OECD, 2012). The modifications were studied using various NMs and proved to produce reproducible results. However, laboratory comparison tests or round robin tests have not yet been conducted and still have to be initiated, if considered necessary by regulatory bodies. The implementation of the proposed modifications in the actual guidelines have to be done at the OECD level and the revision of several test guidelines has been already initiated by the OECD Working Party on Manufactured Nanomaterials.

Approaches that decrease the workload for conducting groups of tests in the overall strategy, or within an individual test should be explored further. This might include options for not conducting a test using decision trees with specific criteria, or a reduced number of treatments and/or concentrations within a protocol, or a short test duration. Avoiding animal testing altogether with *in silico* or *in-vitro* approaches may also alleviate workload concerns. There is also scientific value in these approaches for grouping and ranking (Oomen et al., 2015) and for efficient concern-driven testing strategies (Oomen et al., 2014). Such modifications are currently not considered in the scope of the OECD test guidelines. There may also be data gaps or opportunities for efficiency in the testing strategy for NMs. Here, tests on root elongation of hydroponic plants in hydroponic systems and on fish cells can provide additional information. Descriptions of the tests for NMs are available (S3).

## Conclusions

The adaptation of OECD TGs for aquatic and terrestrial ecotoxicity was proposed for the testing of NMs at an OECD WPMN expert meeting (OECD, 2014). The studies presented here provide significant progress on the necessary adaptations of these OECD TGs. Subjects which are already addressed by further activities such as preparation of stock dispersions and analytical verification were not considered. Firm proposals on the modifications with their justification are now available for methods required for the assessment of NMs in the scope of regulation. They cover the testing of the green algae *Raphidocelis subcapitata* (OECD TG 201, 2011), the daphnid *Daphnia magna* (OECD TG 202, 2004), the fish *Danio rerio* (OECD TG 210, 1992), the sediment organism *Lunbriculus variegatus* (OECD TG 225, 2007), soil microflora (OECD TG 216, 2000; OECD TG 217, 2000) and terrestrial invertebrates *Enchytraeus crypticus* and *Eisenia fetida* (OECD TG 220, 2004; OECD TG 222, 2004). The proposed adaptations include general topics and were developed using Ag and TiO<sub>2</sub> NMs. With the materials applied, two main variants of NMs (ion releasing vs. inert NMs) were addressed. As the modifications of the test guidelines refer to general test parameters (e.g. test duration or measuring principle) we assume that the described approaches and modifications will be suitable for the testing of further NMs with other chemical compositions.

Tests on root elongation of hydroponic plants in hydroponic systems and on fish cells (S3) can fill a gap in the testing strategy with focus on ranking and grouping of NMs as well as for concern-driven test strategies.

## Declaration of interest

The authors report that they have no conflicts of interest. This work was supported by the European Union Seventh Framework Programme [EC-GA No. 263215]. ‘MARINA’.

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