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Integrative test strategy for the environmental assessment of nanomaterials

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Abstract

Main aim of the project was the development of a test strategy on the fate and ecotoxicological effects of synthetic nanomaterials (ENM) in the environment. Furthermore, a tiered risk assessment approach was suggested.

For the test strategy development both, conventional and alternative endpoints were considered, and fate and effects were considered separately. First, the literature was reviewed and suitable test methods were identified. Recommendations for the fate and effects testing were provided. The recommendations consider various levels of test complexity as they are to be used in a tiered risk assessment scheme. Furthermore, the proposed test strategy on effects comprises of three sub-steps: Step 1: decision on ENM to be tested; Step 2: testing; Step 3: use of test results.Exemplarily one aspect of the test strategy for the assessment of effects was investigated comprehensively.

For the risk assessment (RA) approach, a tiered scheme such as are commonly used in the context of precautionary environmental risk assessment was suggested including the use of mathematical models and trigger values to either stop the procedure or proceed to the next tier. Besides risk assessment related aspects, the IME-approach also takes into account the basic idea of life-cycle assessment (LCA).

Kurzbeschreibung

Hauptziel des Projektes war die Entwicklung einer Strategie zur Testung von Verbleib und Wirkung von synthetischen Nanomaterialien (ENM) in der Umwelt. Weiterhin wurde ein gestuftes Verfahren zur Abschätzung des Umweltrisikos von ENM vorgeschlagen.

Zur Teststrategie-Entwicklung sollten neben den konventionellen Endpunkten auch alternative Ansätze in Betracht gezogen werden. Die Aspekte „Verbleib“ und „Wirkung“ wurden getrennt behandelt. Zunächst wurden jeweils anhand einer Literaturrecherche Testmethoden eruiert und Empfehlungen hinsichtlich ihrer Eignung formuliert. Dabei wurde bereits soweit wie möglich berücksichtigt, dass bei einem gestuften Verfahren zur Risikoabschätzung unterschiedlich komplexe Testmethoden eingesetzt werden sollten. Darüber hinaus besteht die vorgeschlagene Effekt-Teststrategie aus drei Unterschritten: Schritt 1: Entscheidung über die Testung von ENM; Schritt 2: Testung; Schritt 3: Anwendung der Ergebnisse. Ein Aspekt der Strategie zur Ökotoxikologie wurde exemplarisch näher experimentell betrachtet.

Beim vorgeschlagenen Verfahren zur Risikoabschätzung handelt es sich um ein gestuftes Verfahren, wie es im vorsorgenden Umweltschutz üblicherweise eingesetzt wird. Das Verfahren enthält des Weiteren mathematische Simulationsmodelle sowie Werte, die entweder ein “STOP” der Prozedur oder ein Beschreiten der nächst-höheren Stufe triggern. Neben den Aspekten der Risikoabschätzung werden auch einige grundlegende Ideen der Lebensweg-Betrachtung (life-cycle assessment, LCA) eingebracht.

Table of Contents

List of Figures

List of Tables

List of Abbreviations and Definitions

CNT	carbon nanotubes
DGT	diffuse gradients in thin films
ECx	effect concentration with x % inhibition
ENM	engineered nanomaterials
ENP	engineered nanoparticles
Intrinsic property:	property of an ENM which not influenced by anyexternal factors such as the amount tested, other test parameter or environmental parameter. The expression is used herein in the sense of an "absolute" property rather than a "comparative" property.
OECD WPMN	OECD Working Party on Manufactured Nanomaterials
PC-parameter	physico-chemical parameter
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
n-PEC	predicted environmental concentration for nanomaterials
n-PNEC	predicted no effect concentration for nanomaterials
OPPTS	Office of Prevention, Pesticides and Toxic Substances
RIP-oN2	REACH Implementation Project on Nanomaterials 2
suffix "ini"	referring to the initial compartment (e.g. n-PECini)
suffix "sec comp"	referring to the secondary compartment (e.g. n-PECsec)
suffix "refined"	referring to a refined value (e.g. n-PECini refined)
TG	test guideline, test guidelines
RQ	risk quotient

1 Summary

1.1 Test strategy

Aim of the project was the proposal for a test and environmental risk assessment strategy for engineered nanomaterials (ENM) addressing the aspects fate and effect.

The proposed IME-test strategy and risk assessment approach is a life-cycle oriented one, and thus considers all stages along the life of the ENM. For each single stage it has to be considered whether there is a potential for the ENM to be released into the environment. Furthermore, the initial environmental compartment the ENM is released into has to be identified. If the release potential is negligible, this particular life-cycle stage needs no further consideration.

In case the ENM is released into the environment its durability in the initial compartment should be screened (tier 0). For that screening any information on the changes or loss of ENM properties is indispensable. In case low durability is ascertained, i.e. the ENM rapidly loses its nano-material properties, the formed chemicals can be handled as conventional chemicals. In case medium to high durability is stated, the first tier of the assessment scheme is entered.

On tier 1, both a fate and effect assessment is performed. The assessment results in a predicted environmental concentration in the initial compartment (n-PEC $_{ini}$) and a predicted no effect concentration (n-PNEC). The prefix "n" is used to characterise the PEC and PNEC as concentrations for "nanomaterials" and the distinction to the bulk material is emphasised.

The deduction of n-PEC $_{ini}$ needs the information on parameters which determine medium to high durability, and also on the amount of the ENM released as well as on the production volume. It furthermore needs a setting of the size of the initial compartment, e.g., the definition of a local or regional scenario as well as other information such as dispersion stability and physicochemical properties of the pristine ENM. The effect assessment is based either on screening tests (in case of low production volume) or on tests using OECD test-guidelines (in case of high production volume). Thus, the effects testing on tier 1 comprises two different levels of complexity. Besides n-PNEC-values a classification and product labelling (CPL) can be suggested on the basis of the effect concentrations.

Comparably to conventional chemicals, a risk quotient (n-PEC $_{ini}$ / n-PNEC) can be formed. In case it is below 1 a tolerable risk for the initial compartment can be assumed. No further sophisticated risk assessment for the initial compartment is needed. In case it is above 1 the risk for the initial compartment might not be negligible and thus, a refinement on tier 2 (n-PEC $_{ini\ refined}$; n-PNEC refined) is needed. Regardless of the risk quotient for the initial compartment, a possible ENM transport to a secondary compartment (n-PEC $_{sec\ comp}$) needs further consideration. The calculation of n-PEC $_{ini\ refined}$ and of n-PEC $_{sec\ comp}$ need experimental input data and modelling. As environmental fate processes of ENM are kinetic processes but no equilibrium processes, respective fate models have to be kinetic ones.

The refined n-PNEC-assessment on tier 2 comprises a higher tier testing, i.e. chronic tests instead of acute tests (e.g. chronic test with plants), the use of further test species resulting in species sensitivity distributions or by more realistic exposure scenarios such as aquatic mesocosm. In case of a likely exposure of a secondary compartment appropriate effect tests have to be considered. The testing and the use of assessment factors result in n-PNEC$_{ini\ refined}$ and n-PNEC$_{sec\ comp}$.

Tier 2 yields a refined risk quotient for the initial compartment (n-PEC $_{ini\ refined}$ / n-PNEC$_{refined}$) and – in case of a likely exposure of a secondary compartment – in a risk quotient for that compartment (n-PEC $_{sec\ comp}$ / n-PNEC$_{sec\ comp}$). As on tier 1 the trigger of 1 is used to either "STOP" or to proceed to a further tier. Tier 3 might comprise an additional even more sophisticated test refinement or risk mitigation.

Additionally to the theoretical considerations leading to the IME test strategy on ENM, one specific topic – namely the appropriateness of the avoidance behaviour test with *Eisenia fetida* (ISO 17512-1) as a screening test on tier 1 – was addressed experimentally.

The suggested test strategy has been developed based on the knowledge of (inter)national publications and discussions, It also takes into account the conclusions made by the OECD WPMN on ecotoxicity and environmental fate which met in Berlin in January 2013. Additionally, the test strategy was compared with proposals presented by German Competent Authorities and of the REACH Implementation Project on Nanomaterials (RIP-oN2). These strategies focus on specific aspects whereas the IME test strategy is more comprehensive.

1.2 Effect assessment

1.2.1 General aspects

ENM to be tested

We propose that ENM with PC-parameters indicating no ecotoxicity in any environmental compartment (water, sediment, soil) without any doubt do not have to be tested. Furthermore, testing is not required for ENM for which a direct or indirect exposure of the environment can be excluded. ENM which may be toxic, distributed in the environment and whose production volume exceeds a threshold value should be tested as well as the nano-form of ecotoxic bulk material and ENM with expected active properties (independent of the production volume). The threshold value still needs to be defined in expert groups or committees. There should be one threshold value for all ENM which is based on one, rather low production volume. Small production volumes below the threshold value may be of less relevance with respect to environmental exposure and thus environmental damage. However, to detect highly toxic ENM with a small production volume and which are not obviously toxic due their PC-properties, we recommend the performance of a screening test for such ENM.

If ENM are covered by specific legislations where production volumes are not considered (e.g. ENM used as biocides or pesticides), the aspect "production volume" can be neglected in the presented test strategy. These ENM should be tested according to the requirements of the respective legislation, but should additionally consider the nanospecific aspects proposed within this test strategy (e.g. selection of tests: chronic tests instead of acute tests).

Screening test

The screening test and the interpretation of the results still have to be agreed on. The screening test should be used as a kind of analytical device. Therefore, the test need to have a high sensitivity, the indicator function concerning effects on populations is less important. Criteria for such tests should be (I) easy performance, (II) short test duration, (III) sensitivity comparable to sensitivity of the standardised endpoints to avoid too many „false" positive or "false" negative results. High throughput assays covering various endpoints of high diversity or modelling might be suitable. The suitability of such methods and procedures for the initial examination has to be investigated and the most reliable procedures need to be further developed. If the results indicate significant toxicity, the test program applicable to those ENM whose production volumes exceed the threshold value (see below) has to be performed.

Test systems for ENM with production volumes exceeding the threshold value or with ecotoxic bulk material or ENM with expected active properties

So far, there are considerable knowledge gaps with regard to the sensitivity of aquatic tests in comparison to terrestrial tests. It cannot be excluded that terrestrial tests are of comparable sensitivity or even more sensitive than aquatic tests as exposure can differ. In aquatic tests the exposure concentrations and availability can decrease due to agglomeration and – depending on the test conditions - sedimentation. Sedimentation will result in increased exposure for sediment organisms. In contrast, in soil the exposure of the organisms is comparatively stable. Additional agglomeration during the test period is not expected. Therefore, we recommend a test program which includes equivalently the three compartments surface water, sediment, soil. We assume that ENM will preferentially enter the sediment compartment via the water phase. Therefore, we recommend the test on sediment organisms performed using spiked water instead of spiked sediment to simulate realistic exposure. Degradability of most ENM is limited due to their inorganic condition and long-term exposure is expected. Therefore, we prefer chronic tests (if available) instead of acute tests. We suggest following test program for ENM:

a) Aquatic tests
Daphnids: OECD TG 211; algae: OECD TG 201; fish: OECD TG 210 "Fish, Early-life Stage Toxicity Test"

b) Sediment test
Chironomids: OECD 219 (spiked water phase) or Lumbriculus: OECD TG 225 (so far, a TG for the Lumbriculus test using spiked water is not available and a development is recommended)

c) Terrestrial tests
Microflora: OECD TG 216 using an inorganic nitrogen source instead of an organic one; earthworms: OECD TG 222; plants: OECD TG 208
(Explanation for inorganic nitrogen source in a test according to OECD TG 216: Released ions tend to sorb to the additional organic nitrogen source thus reducing their bioavailability. We instead recommend the use of an inorganic nitrogen source or a test on potential ammonium oxidation according to ISO Guideline 15685(2004).)

The metric to be used for the presentation of the toxicity is still discussed. Besides mass also size/surface area of ENM and particle number may be suitable. We recommend the presentation of the results on mass basis to allow comparability with the results obtained with the bulk material. In addition, the surface area, the particle number, the particle size distribution of the ENM and the methods used for the determination should be mentioned. If required, the results can be recalculated using the metric "surface" or "particle number".

In several terrestrial and aquatic tests with various ENM a plateau with a maximum effect below 100 % is observed instead of concentration-effect relationships with a maximum of 100 % effect. Therefore, we recommend the investigation of several concentrations per test instead of limit-tests with only one test concentration to receive information whether comparable effects are achieved at lower test concentrations.

The test conditions described in the test guidelines usually do not support the investigation of photocatalytic activity. Simulated sunlight can increase ecotoxicity of photocatalytic active ENM and possibly also of further ENM types. We recommend aquatic tests to be performed according to the guidelines but also with simulated sunlight for photocatalytic ENM. The tests with conventional lighting are recommended to link the results to results obtained by applying the current test guidelines. The most sensitive result, independent of the illumination conditions, should be used for the characterisation and assessment of ENM. In addition, the knowledge on the effect of illumination on ecotoxicity of non-photocatalytic active ENM has to be improved.

Use of test results

The test results can be used to describe the *intrinsic* properties of ENM. Additionally, *classification and labelling* as well as an initial environmental risk assessment can be performed. For each purpose only the relevant test results need to be used. With respect to ecotoxicity, classification and labelling should address the most endangered environmental compartment.

For *risk assessment* PNEC values are required. They are calculated using appropriate effect data and assessment factors. There are no indications that assessment factors differing from the existing ones are needed for ENM. The PNEC values have to be compared with environmental concentrations (PEC). However, so far, it is difficult, if not impossible, to characterise ENM in solid matrices such as soil and sediment. As modifications of the ENM are difficult to determine and the role of ion toxicity for ion releasing ENM is not yet clarified, we recommend the definition of a n-PEC_{ini}. The PEC-values, specific for ENM and hence marked by the "n-", refer to the concentration of the pristine ENM. With increasing state of knowledge on the influence of modifications or with improved methods concerning characterisation of ENM, the term "n-PEC_{ini}" can be adapted. We propose to use the same approach for the aquatic compartment to achieve comparability.

So far, the knowledge concerning the effect of aging on toxicity of ENM is limited. If the knowledge gained by further experiments shows that aging increases ecotoxicity, we recommend the categorization of ENM in (I) materials affected by aging and (II) ENM which are not affected. ENM should be tested twice if it is assumed that ecotoxicity increases by an aging process. We recommend the testing of the pristine ENM as well as of the material aged in the test medium. Testing of pristine ENM is considered to be sufficient if ecotoxicity is expected to decrease or not to be affected by aging.

1.2.2 Specific aspect: Alternative test systems in the test strategy for the assessment of ENM

The appropriateness of the OECD test guidelines as well as other guidelines for nanomaterials have been reviewed and it is explained that the endpoints are adequate and relevant also for ENM. Some modifications of the test procedures are required and currently the existing OECD test guidelines are updated and new ones are drafted.

Besides the application of the standardized test methods, alternative test methods and endpoints are published for the assessment of ENM. So far, it is not obvious whether these endpoints provide additional information within the framework of regulation justifying the integration in the test strategy for ENM. In the project a literature review on alternative test methods such as behavior, nutritional performance, indicators for oxygen stress, haematology, histology, genotoxicity, cytotoxicity, neurotoxicity, immunotoxicity, bioaccumulation and biodiversity was performed. Following conclusions were drawn:

1. The conventional endpoints used for risk assessment are selected with respect to the protection of populations and cover parameters such as reproduction, mortality, growth. Individuals are not considered. The alternative parameters reviewed in the literature usually address less complex reactions (e.g. determination of specific enzymes or gene activities) often resulting in an increased sensitivity. It is not always obvious whether an effect detected by a sensitive additional endpoint (e.g. indicators for oxygen stress) has an impact on the population level or indicates a compensation measure of the organism. Nevertheless, every additional parameter can provide additional information on ecotoxicity of ENM and can support the assessment. In any case, in research, alternative endpoints play a major role by increasing the knowledge on the mode of action of ENM and improving their assessment. However, for regulation, the advantage compared to the current procedure could be limited. However, research on the suitability of alternative endpoints is ongoing and final conclusions are not yet possible.
2. There can be some specific effects which are not detected with the conventional endpoints but which might have an impact on the population level and as such might be of relevance for assessing the hazard of ENM for regulatory purposes.
    a. Immunotoxicity / Genotoxicity
    The knowledge on the significance of effects on immunotoxicity and genotoxicity caused by ENM in-vitro and on the population level should be improved. Furthermore, the results have to be compared with the results obtained within the scope of studies on human toxicology. Based on this information it can be agreed on whether these parameters are a suitable addition to the ecotoxicological test strategy or whether the information from human toxicological studies is sufficient.
    b. Bioaccumulation
    So far, the knowledge on PC-parameters indicating accumulation of ENM is limited. To get information on the accumulation potential of ENM we recommend a pragmatic procedure. We propose to determine the ENM concentration in suitable test organisms of the ecotoxicological tests (e.g. terrestrial and aquatic oligochaetes, daphnids, fish embryos and plants) at the end of the incubation period. By this procedure, first information on uptake of ENM can be achieved. If more detailed results are required specific studies on bioaccumulation can be performed considering the discussions of the OECD expert meeting (Kühnel & Nickel, 2014) and – if already available – specific guidance on accumulation of ENM. Furthermore,

the screening procedure on accumulation can be used to identify PC-parameter indicating bioaccumulation.

c. Multi-generation-tests
We cannot exclude that effects become more pronounced if multi-generation-tests are performed. Additionally, recovery studies can provide interesting information. However, for regulatory testing the experimental effort connected to these kind of studies should not be mandatory. Uncertainty is considered in the risk assessment, e.g. by assessment factors. Furthermore, we assume that the information obtained by multi-generation-tests and recovery studies is not nano-specific. It should also apply for conventional chemicals and for these chemicals the current risk assessment procedure has been successfully applied since many years.

d. *Further test organisms*
ENM agglomerate in aquatic systems, and increased concentrations in the sediment are expected. The standardized test organisms *Chironomus riparius* and *Lumbriculus variegatus* develop in the sediment. It cannot be excluded that organisms living and grazing on the sediment as well as floated submerged, aquatic macrophytes are exposed to a higher extend compared to the standard test organisms. We recommend that the sensitivity of potential suitable organisms (sediment organisms, aquatic macrophytes) and of the standard test organisms (*C. riparius, L. variegatus, Lemna minor*) are compared to decide on the suitability of further test organisms or the replacement of traditional organisms for the testing of ENM.

e. Behavioural tests
In the reviewed literature, behavioural tests revealed to be quite sensitive. However, so far, the information on the applicability on a wide range of ENM is limited. To extend the knowledge, the behavioural test with earthworms was studied in more detail in the experimental section of this project. For a wide spectrum of conventional chemicals, it was demonstrated that the sensitivity of the avoidance behaviour with *Eisenia fetida* (ISO 17512-1) reaches or increases the sensitivity of reproduction tests (OECD 222).
Five ENM from the OECD-Sponsorship Programme (CeO_2: NM-211, NM-212, ZnO: NM-111, CNT: NM-403, Ag: NM-300K) and soluble $AgNO_3$ were investigated in both tests. The selected substances differ in their solubility and stability.
It became obvious that the avoidance test with its short incubation period can provide interesting information. A general application as screening test is not recommended. Depending on the ENM-properties different information can be achieved.

- The test provides information on the ecotoxicity of ion releasing ENM and can be used for the ranking of such substances. The test can underestimate ecotoxicity if it is performed immediately after spiking with ENM which are quite poorly soluble or which become toxic by aging.
- The test can be used to give first indications on the fate of ENM in the complex matrix soil. Currently analytical methods are missing to

describe the ENM in a complex environmental medium such as soil or sediment. However, care must be taken in the interpretation of the results. Exemplarily, it has to be considered that reduced ecotoxicity can be the consequence of decreased bioavailability due to sorption.

- We recommend further research to clarify the observed discrepancy between effect and concentrations (missing concentration-effect relationship for poorly water soluble ENM)

1.3 Fate assessment

1.3.1 General aspects

In order to select appropriate parameters, test design and test methods for a test strategy on environmental fate of ENM, recent literature was compiled for environmental fate related key words which then were allocated to the parameter "solubility", "partitioning", "stability in the (aquatic) environment", "transformation", "degradation", "mobility and transport in porous media", "sorption / desorption to soil, sediment, and sludge", "bioaccumulation", "PEC-assessment, PEC-models" and "risk assessment approaches".

Solubility and partitioning

Several citations show that OECD-test guidelines on solubility (OECD 105, 107, 117) and partitioning (OECD 117, 107, 123) have been slightly modified to be used with carbon based ENM such as fullerenes and CNTs. However, such modified test guidelines might give valuable results, in case ENM of comparable nature (e.g. fullerenes, CNTs) are tested with identical methodological approaches. In this case the results might be used for a comparison and ranking of structurally comparable ENM. They are not suitable to elucidate the ENM intrinsic properties. It furthermore is commonly accepted that guidelines which are based on partitioning processes are not suitable for ENM since non-equilibrium processes between ENM and the surrounding media occur. The most promising test approaches are those which consist of complex systems mimicking the environmental conditions. Such approaches consider the complex nature of ENM and their complex interactions with the environment.

Stability in the (aquatic) environment

Under that topic also agglomeration / aggregation as well as colloid stability are covered. Several articles deal with the influence of the aquatic environmental conditions such as pH-value, ionic strength, humic acid content on the stability of ENM in the aquatic environment. It can be concluded that further research is required to investigate the interactions between the aquatic environment and ENM. Experimental approaches which are based on modified standard test guidelines yield valuable results for comparing the behavior of various nanoparticles in aquatic systems. However, they do not result in the description of intrinsic properties. Modifications of standard tests, e.g. by the addition of natural organic matter sampled from a natural river or creek, should be considered. Even more useful to describe the stability of ENM in natural aquatic systems is the use of media mimicking the respective environmental conditions. This can in particular be achieved by using a complex laboratory test system such as model waste water treatment plants or fresh water mesocosms. However, though the conclusion on the need of complex systems is commonly accepted in the scientific community none of the cited references explicitly deals with this issue in detail.

Mobility and transport in porous media, sorption / desorption to soil, sediment, and sludge

Most articles deal with the mobility and transport of ENM in porous media and soil, rather than in the aqueous compartment, although sedimentation is an important process in the water.

ENM stability during experiments and their characterization and quantification is usually addressed. It is well known that the size and size distribution are of major importance for the environmental behavior of ENM and should thus be characterized and well documented in each experiment. It is recommended that besides size or size range a further characterization of ENM shape or nature and surface properties should be made.

It is well known that the concept of sorption which is based on distribution coefficients, and which is of major importance for the description of solutes transport in soil, is not appropriate for ENM. However, ENM association with soil is a non-equilibrium process, as it is also in other environmental compartments. Processes, which in addition have to be considered, are dispersion, aggregation, deposition and re-mobilization. It furthermore is evident, that most experimental set-ups might affect the form in which nanomaterials occur. Thus, the results obtained are not representative for the materials´ behavior in the real environment. An example: the soil to solution ratio which is used in batch experiments according to OECD 106 is by far lower compared to the real environment and thus provokes aggregation or dissolution of the nanomaterials which does not occur in the environment.

Only in a few studies has been tried to adopt and modify OECD 106 which has mostly failed due to the limited interpretability of the results. Forouzangohar et al. (2011), for example, investigated the importance of hydrophilic forces in controlling the sorption behavior of fullerene particles which were stabilized in water dominated solvent mixtures. The authors questioned the general validity of partitioning mechanisms and Koc-modelling approaches in describing and estimating the sorption of nC60 particles in soil. However, it was stated that it may be valid for more un-polar dominated solvent mixtures, e. g, containing more than 60 % ethanol. Kiser et al. (2012) investigated the sorption of ENM to activated sludge as required by OPPTS. However, sorption of ENM has to be determined by the use of fresh sludge, and thus, the OPPTS methods as it stands needs to be modified. As long as differently treated sludge surfaces are compared, the method is appropriate. However, in case the ENM inherent property is to be determined, the method is questionable as the results depend on the clean-up methodology. Furthermore, solution ratio (see above) has to be taken into account.

In general it can be concluded that the use of experiments mimicking environmental situations is most appropriate. In particular these are soil column experiments, e.g. as described in OECD 312. Most importantly, various techniques, in particular analytical techniques, should be combined in order to obtain a comprehensive and reliable picture of the ENM mobility in porous media.

Bioaccumulation in aquatic organisms

Few references are available describing the applicability of bioaccumulation tests for ENM. Generally, the determination of bioaccumulation needs to account for the fact that uptake- and distribution-processes are kinetically driven.

Bioaccumulation in soil and sediment living organisms

Tests as laid down in OECD 315 and 317 are generally applicable The bioaccumulation factor (BAF) is an appropriate endpoint, however, specific guidance is needed on how to perform the test and interpret the results. Wet spiking is mostly preferable but there may be reasons why not. Purged and unpurged worms should be investigated.

PEC-assessment, PEC-models

Environmental fate processes of ENM which are mostly aggregation, transformation and sedimentation processes are non-equilibrium but kinetic processes, because ENMs do not reach thermodynamic equilibrium but are present in the environment as unstable suspensions. Thus, conventional distribution models based on equilibrium processes such as the fugacity models developed by Mackay are not applicable. ENM fate models have to be designed and evaluated which are capable to incorporate the environmental complexity to predict realistic environmental concentrations of ENM. Furthermore, the use of kinetic models is essential in PEC-assessment.

Quite often reliable data are missing, e.g., on the quantity of emissions into the environment during production and usage. This situation can be overcome to some extend by using probabilistic density functions.

Risk Assessment (RA) approaches

Only a few references deal with the risk assessment of ENM. These comprise:

- Comparison of RA of conventional substances and RA of ENM.
- Dealing with uncertainties and limited input information.
- Integration of ENM alteration and transformation in the RA.

Uncertainties regarding the potential impacts and risks associated with ENM were discussed by Adam et al. (2014). The authors combined life-cycle assessment (LCA) and RA approaches. Because high uncertainties remain concerning the fate and effects of ENM probabilistic approaches are needed. A Bayesian network was used. Nowack et al. (2012) concluded that risk due to ENM cannot be determined exclusively for pristine ENM but has to consider alterations and transformation in the environment.

1.3.2 Environmental fate: overall conclusions based on OECD WPMN and literature search

From the literature screening and the conclusions drawn by the OECD WPMN most important conclusions are:

- Published results have to be evaluated carefully with respect to the reliability and robustness of the analytical methods applied. Publications without exact description of the applied analytical methods should not be taken into account for use in the integrative test strategy.
- In particular for the determination of size and size distribution a set of different analytical methods should be applied to obtain the most comprehensive information. But generally, the use of more than one analytical method seems to be useful to account for the complex nature of ENM.

- Most experimental set-ups are likely to affect the form in which ENM occur and might yield a result that is not representative of the behavior under realistic environmental conditions.
- Tiered testing and risk assessment strategies are appropriate to account for the complex nature of ENM. They also account for the fact that ENM might be short lived in the environment. In that case, risk assessment should be based on the "free", conventional substance (e.g. on the free metal ion or the dissolved active ingredient in case of nanopesticides) – if that occurs.
- Risk assessment should be based on information on aged rather than on pristine ENM only since that accounts for the durability of the ENM in the environment.
- The more complex the considered environment matrix is, the more environmental parameters interact with ENM which themselves are of complex nature. Consequently, the experimental test design needs to reflect this: The more complex the tested compartment is, the more should the experimental set-up mimic the real environment.
  E.g., on a first tier, fate and degradation in water can be investigated relatively easily by the use of a "standard water" and the determination of dissolution (new TG needed), dispersion stability (new TG needed), and the measurement of the stability of the organic coating (new or modified TG needed). On higher tiers, microcosm or mesocosm studies might be valuable tools.
  In contrast, fate and degradation in soil, even on the first tier, should be investigated using a soil leaching study (OECD 312) mimicking the vertical transport through the soil. By using an aged soil leaching study, even the transformation can be taken into account.
- The development of new test methods or the interpretation of existing methods should be related to the protection target.
  It should – for example – be discussed as to whether a modified OECD 106 (sorption to soil) is even needed or whether it can completely be skipped and replaced by OECD 312 (soil column leaching). In this case, the aim of protection is groundwater protection. That means, that the portion of ENM which is eluted from the column is relevant for groundwater exposure and sorption constants are not all used for the groundwater risk assessment.
- It is worth to combine risk assessment and life-cycle assessment approaches.
- n-PEC assessment has to face the fact that fate processes (mainlyaggregation, transformation and sedimentation processes) are kinetically dominated and non-equilibrium processes. Respective models have to be used.
- One approach to account for lacking information on input parameters for PEC models is to deal with uncertainties.

2 Zusammenfassung

2.1 Teststrategie

Hauptziel des Projektes war die Entwicklung einer Strategie zur Testung und Risikobewertung von Verbleib und Wirkung von synthetischen Nanomaterialien (ENM) in der Umwelt.

Die vorgeschlagene IME-Teststrategie sowie der Ansatz zur Risikobeurteilung berücksichtigen den gesamten Lebenszyklus der ENM. Für jeden Abschnitt des Lebenszyklus ist zunächst zu überprüfen, ob eine Freisetzung des ENM erfolgen kann. Ist dies der Fall, ist das Umweltkompartiment zu identifizieren, in das das ENM zunächst freigesetzt wird (= Initialkompartiment). Ist das Freisetzungspotential vernachlässigbar, muss dieser spezifische Lebenszyklusabschnitt nicht weiter betrachtet werden.

Im Fall einer Freisetzung des ENM ist dessen Beständigkeit zu beurteilen (Stufe 0, tier 0). Hierbei sind alle Informationen zu potentiellen Veränderungen und dem Verlust ENM-spezifischer Eigenschaften zu berücksichtigen. Gehen die ENM-spezifischen Eigenschaften rasch verloren, kann die entstehende Substanz als konventionelle Chemikalie betrachtet und entsprechend beurteilt werden. Im Fall einer signifikanten Beständigkeit als ENM, schließt sich die Stufe 1 (tier 1) in der Beurteilung an.

Tier 1 umfasst sowohl den Aspekt Verbleib als auch den Aspekt Effekt (Wirkung), wobei zum einen ein n-PEC (*predicted environmental concentration*) $_{ini}$ als auch ein n-PNEC (*predicted no effect concentration*) errechnet wird. Das Präfix „n“ macht dabei deutlich, dass es sich um Werte für ENM handelt und unterstreicht damit den Unterschied zur Nicht-Nanoform des entsprechenden Materials („bulk material“). Die Bezeichnung „ini“ zeigt an, dass es sich um die Umweltkonzentration für dasjenige Kompartiment handelt, in das die ENM zunächst eingetragen werden (Initialkompartiment).

Für die Ableitung eines n-PEC $_{ini}$ werden Informationen zur Beständigkeit der ENM in der Umwelt, zur Dispersionsstabilität, Lösungskinetik, zur Veränderung des Coatings, zu physiko-chemischen Eigenschaften, zur freigesetzten Menge und zum Produktionsvolumen benötigt. Auch die Größe des Initialkompartimentes, in das die ENM eingetragen werden, ist festzulegen. So muss beispielsweise entschieden werden, ob ein lokales oder ein regionales Szenarium zu berücksichtigen ist. Für die Effektbeurteilung sind bei Stufe 1 Untersuchungen mit zwei Komplexizitätsstufen zu unterscheiden. Über die Anwendung entscheidet das Produktionsvolumen, wobei der Schwellenwert noch festzulegen ist. Bei Produktionsvolumina, die den Schwellenwert übersteigen, ist eine umfassende Testung auf Basis der OECD-Standardtests durchzuführen. Im Fall von niedrigen Produktionsvolumina basiert die Beurteilung der Effekte auf Screeningtests, um ENM mit einem hohen Toxizitätspotential zu identifizieren. Die Effektwerte einer umfassenden Testung können sowohl zur Berechnung von n-PNEC-Werten als auch zur Klassifizierung und Kennzeichnung (CLP) herangezogen werden.

In Übereinstimmung mit der Vorgehensweise bei konventionellen Chemikalien ist ein Risikoquotient aus n-PEC $_{ini}$ / n-PNEC zu berechnen. Liegt er unter eins, ist von einem tolerierbaren Risiko für das entsprechende Umweltkompartiment auszugehen. Übersteigt er den Wert von eins, ist ein Risiko nicht auszuschließen, so dass eine Verfeinerung der Risikoabschätzung mit Steigerung der Realitätsnähe vorzunehmen ist (Stufe 2, tier 2; n-PEC $_{ini\ refined}$; n-PNEC $_{refined}$). Unabhängig vom Ergebnis der Risikoabschätzung auf der Stufe 1 ist ein potentieller Transport in ein weiteres Umweltkompartiment zu überprüfen (n-

PEC $_{sec\ comp}$). Sowohl für die Berechnung von n-PEC $_{ini\ refined}$ als auch von n-PEC $_{sec\ comp}$ werden weitere experimentelle Input-Daten bzw. Modellierungsansätze benötigt. Bei ENM liegen kinetische und keine Gleichgewichtsprozesse vor, so dass für die Modellierung entsprechende kinetische Modelle heranzuziehen sind.

Die Verfeinerung des n-PNEC in Stufe 2 kann durch weitere chronische Tests (z.B. chronischer Pflanzentest), die Verwendung weiterer Testarten und damit die Berechnung einer Spezies-Sensitivitätsverteilung oder die Anwendung von realitätsnäheren Expositionsszenarien (z.B. aquatische Mikrokosmen) erfolgen. Zeigen die Ergebnisse der Verbleibsbetrachtungen, dass die ENM in ein weiteres Kompartiment neben dem primären Eintrittskompartiment verlagert werden können, sind ggfs. auch hier weitere Untersuchungen notwendig. Die Testung und die Verwendung von Bewertungsfaktoren resultieren dann in PNEC $_{ini\ refined}$ und PNEC $_{sec\ comp}$.

Das Ergebnis der Stufe 2-Testung besteht aus einem verfeinerten Risikoquotienten für das Initialkompartiment (n-PEC $_{ini\ refined}$ / PNEC $_{refined}$) und – im Fall eines möglichen Eintrages in ein zweites Kompartiment –auch aus einem Risikoquotienten für dieses Kompartiment (n-PEC $_{sec\ comp}$ / PNRC $_{sec\ comp}$). Wie bereits in Stufe 1 wird auch in Stufe 2 ein Trigger von 1 herangezogen. Bei einem Unterschreiten des Schwellenwertes kann die Beurteilung beendet werden. Ein Überschreiten führt in einer Stufe 3 entweder zu weiteren experimentellen Untersuchungen mit noch weitergehender Realitätsnähe bzw. zu Risikominderungsmaßnahmen.

Neben der rein theoretischen Betrachtungen zur Teststrategie wurde ein Aspekt – die Eignung des Regenwurmvermeidungstest (ISO 17512-1) als Screeningtest in Stufe 1 - experimentell näher betrachtet.

Die vorgeschlagene Teststrategie wurde auf Basis von (inter)nationalen Publikationen und Diskussionen sowie auf Basis der Vorschläge aus dem OECD WPMN Workshop zu Ökotoxikologie und Umweltverhalten (Berlin, Januar 2013) entwickelt. Darüber hinaus wurde ein Vergleich mit dem Vorschlag der Deutschen Bundesoberbehörden sowie mit dem des *REACH Implementation Project on Nanomaterials* (RIP-oN2) vorgenommen. Dabei wurde deutlich, dass die IME-Teststrategie für Nanomaterialien deutlich umfassender ist, während die beiden anderen Vorschläge jeweils spezifische Aspekte betrachten.

2.2 Effektbeurteilung

2.2.1 Generelle Aspekte

Zu testende ENM

Wir schlagen vor, dass ENM mit physikalisch-chemischen Parametern, die eindeutig darauf hinweisen, dass keine signifikante Toxizität in einem der Umweltkompartimente (Wasser, Sediment, Boden) zu erwarten ist, nicht getestet werden müssen. ENM, für die eine direkte oder indirekte Exposition der Umwelt ausgeschlossen werden kann, sollten ebenso keine Berücksichtigung in der Teststrategie finden. ENM, für die eine Ökotoxizität nicht ausgeschlossen werden kann und deren Produktionsvolumen über einem Schwellenwert liegt, sollten ebenso untersucht werden, wie ENM, bei denen die Nicht-Nanoform des entsprechenden Materials („bulk material“) ökotoxische Eigenschaften aufweist. Bei letzteren sollte die Produktionsmenge keine Rolle spielen. Wir schlagen vor, dass durch ein Expertengremium ein Schwellenwert für alle ENM festgelegt wird. Niedrige Produktionsvolumina unter dem Schwellenwert könnten von geringerer Bedeutung im

Hinblick auf eine potentielle Umweltexposition und daher potentielles Umweltrisiko sein. Um jedoch hoch toxische ENM mit geringem Produktionsvolumen zu erkennen, schlagen wir vor, dass alle ENM mit einem geringem Produktionsvolumen und für die ein hohes ökotoxisches Potential nicht per-se erkennbar ist, einen Screeningtest durchlaufen.

Fallen ENM unter eine spezifische Gesetzgebung, bei der das Produktionsvolumen nicht berücksichtigt wird (z.B. ENM, die als Biozide oder Pflanzenschutzmittel eingesetzt werden), kann der Aspekt „Produktionsvolumen" entfallen. Die Testung dieser ENM sollte der jeweiligen Rechtssprechung entsprechen, jedoch zusätzlich die nanospezifischen Aspekte berücksichtigen, die in der vorliegenden Teststrategie empfohlen werden (z.B. Testauswahl: verstärkt chronische Tests statt akuter Tests)

Screeningtest

Über den Screeningtest und die Interpretation der Ergebnisse muss noch Einigung erzielt werden. Dieser Test soll als eine Art analytisches Instrument dienen und muss daher eine ausreichende Sensitivität aufweisen, wohingegen die Indikatorfunktionen auf Populationsebene keine Priorität besitzt. Folgende Kriterien sollte er erfüllen: (I) leichte Durchführbarkeit, (II) kurze Testdauer, (III) eine Sensitivität vergleichbar zu der der standardisierten Endpunkte zur Vermeidung falsch positiver oder falsch negativer Ergebnisse. „High-throughput tests", die verschiedenste Endpunkte abdecken oder Modellierung könnten geeignet sein. Die Eignung möglicher Verfahren ist noch zu untersuchen und die am vielversprechendsten Methoden sind weiterzuentwickeln. Zeigen die Ergebnisse, dass eine signifikante Toxizität vorhanden ist, ist das Testprogramm für diejenigen ENM mit einer Tonnage über dem Schwellenwert anzuwenden (s. unten).

Testsysteme für ENM mit Produktionsmengen, die den Schwellenwert übersteigen oder bei denen die entsprechende Nicht-Nanoform des Materials (bulk material) ökotoxische Eigenschaften aufweist oder ENM mit erwarteten aktiven Eigenschaften

Noch ist die allgemeine Sensitivität aquatischer Testverfahren im Vergleich zu terrestrischen Methoden nicht geklärt. Es kann nicht ausgeschlossen werden, dass die terrestrischen Verfahren eine vergleichbare Sensitivität zu den aquatischen Methoden aufweisen oder diese sogar übersteigen, da sich die Exposition unterscheidet. So kann in aquatischen Tests die Expositionskonzentration und Verfügbarkeit aufgrund von Agglomeration und – abhängig von den Testbedingungen - Sedimentation abnehmen, wohingegen die Organismen bei den terrestrischen Tests während der gesamten Testlaufzeit einer vergleichsweise stabilen Konzentration ausgesetzt sind. Eine weitere Agglomeration der ENM wird nicht erwartet, da es sich um statische Versuchsbedingungen handelt. Sedimentation resultiert dagegen zu einer erhöhten Exposition von Sedimentorganismen. Wir schlagen daher ein Untersuchungsprogramm vor, dass alle drei Umweltkompartimente, Oberflächenwasser, Sediment, Boden, umfasst. Wir gehen davon aus, dass ENM vorwiegend über die Wasserphase in die aquatische Umwelt und damit auf das Sediment gelangen und schlagen daher vor, die ENM im Test über die Wasserphase zuzugeben, anstelle in das Sediment zu applizieren. Hierdurch sollten realistischer Umweltbedingungen simuliert werden. Ein Abbau der ENM ist aufgrund ihrer anorganischen Natur limitiert, sodass eine Langzeitexposition der Umweltorganismen erwartet wird. Wir empfehlen daher schwerpunktmäßig chronische anstelle von akuten Tests, woraus sich folgendes Testprogramm ergibt:

a) Aquatische Tests
   Daphnien: OECD TG 211; Algen: OECD TG 201; Fisch: OECD TG 210 "Fish, Early-life Stage Toxicity Test"

b) Sedimenttests
Chironomiden: OECD 219 (*spiked water phase*) oder Lumbriculus: OECD TG 225 (bislang steht noch kein Test mit Applikation in die Wasserphase zur Verfügung, so dass dessen Entwicklung angeregt wird)

c) Terrestrische Tests
Mikroflora: OECD TG 216 Verwendung einer anorganischen N-Quelle anstelle einer organischen; Regenwurm: OECD TG 222; Pflanze: OECD TG 208 (Erklärung für die anorganische N-Quelle im Test gemäß OECD TG 216: Freigesetzte Ionen scheinen an die zusätzliche organische N-Quelle zu binden, wodurch die Bioverfügbarkeit reduziert wird. Wir empfehlen daher die Verwendung einer anorganischen N-Quelle oder die Durchführung der potentiellen Ammoniumoxidation nach Richtlinie ISO 15685 (2004).)

Die Maßeinheit, in der die Ergebnisse angegeben werden sollen, ist noch in der Diskussion. Neben der üblichen Angabe in Form einer Masse, wird auch die Angabe über Größe / Oberfläche der ENM und als Partikelanzahl diskutiert. Wir empfehlen, die Ergebnisse in Form von Masse anzugeben, da dann ein direkter Vergleich mit dem entsprechenden Material in Nicht-Nanoform (bulk material) möglich ist. Bei Bedarf, kann das Ergebnis in jede weitere Maßeinheit umgerechnet werden. Hierfür müssen die Oberfläche, die Partikelanzahl sowie, für eine korrekte Interpretation, die Partikelgrößenverteilung und die Bestimmungsmethoden bekannt sein.

In verschiedenen terrestrischen und aquatischen Tests mit unterschiedlichen ENM wurden Sättigungskurven mit einer Maximalwirkung unter 100 % anstelle von Konzentrations-Wirkungs-Beziehungen mit einer Maximalwirkung von 100 %berichtet. Wir empfehlen daher, ENM immer in mehreren Konzentrationen zu untersuchen und keine Limit-Tests mit nur einer Testkonzentration durchzuführen. Bei der Untersuchung von nur einer Testkonzentration ist unbekannt, ob vergleichbare Wirkungen auch bei niedrigeren Konzentrationen erzielt werden, was die Bewertung von ENM beeinflusst.

Die in den Testverfahren beschriebenen Bedingungen unterstützen in der Regel eine Betrachtung der photokatalytische Aktivität nicht. Simuliertes Sonnenlicht kann die Ökotoxizität von photokatalytischen ENM, aber auch von anderen ENM steigern. Wir empfehlen daher, aquatische Tests sowohl unter herkömmlichen Bedingungen als auch mit simuliertem Sonnenlicht durchzuführen. Die Untersuchung unter Standardbedingungen ermöglicht es, die Ergebnisse mit anderen zu vergleichen, die gemäß Richtlinie durchgeführt wurden. Das sensitivste Ergebnis – unabhängig von der Beleuchtung – sollte für die Beurteilung von ENM herangezogen werden. Darüber hinaus sollten die Kenntnisse über die Auswirkung von simuliertem Sonnenlicht auf die Ergebnisse von nicht-photokatalytisch aktiven ENM verbessert werden.

Verwendung der Ergebnisse

Die Ergebnisse können zur Beschreibung der intrinsischen Eigenschaften der ENM genutzt werden. Darüber hinaus lassen sie sich für Einstufung und Kennzeichnung sowie zur Risikobeurteilung heranziehen. Für jede Zielrichtung müssen nur die notwendigen Resultate verwendet werden. Einstufung und Kennzeichnung im Hinblick auf die Ökotoxizität sollte sich auf das am stärksten gefährdete Umweltkompartiment beziehen.

Für die Risikobeurteilung werden PNEC-Werte benötigt, die unter Berücksichtigung von Bewertungsfaktoren errechnet werden. Es gibt keine Hinweise, dass die herkömmlichen Bewertungsfaktoren nicht auch für die Beurteilung von ENM herangezogen werden können.

Die PNEC-Werte müssen mit Umweltkonzentrationen (PEC) verglichen werden. Bislang ist nur ansatzweise möglich, ENM in komplexen Umweltmedien wie Boden und Sediment zu erfassen und zu charakterisieren. Somit ist es schwierig, Veränderungen von ENM zu erkennen. Ferner ist die Rolle freigesetzter Ionen im Vergleich zur Partikeltoxizität der entsprechenden ENM schwer zu fassen. Wir empfehlen daher, einen n-PEC $_{ini}$ zu verwenden. Die für ENM spezifischen und daher mit einem Präfix „n“ versehenen PEC-Werte beziehen sich auf die Konzentrationen des Ausgangs-ENM (pristines ENM). Mit steigendem Wissen bezüglich der Veränderung von ENM und den Folgen für die Ökotoxizität bzw. mit verbesserten Methoden zur Charakterisierung der ENM in komplexen Medien, kann der Begriff „n-PEC $_{ini}$“ angepasst werden.

Noch ist das Wissen zu den Folgen einer Alterung für die Ökotoxizität begrenzt. Zeigt sich aufgrund weiterer Untersuchungen, dass eine Alterung von ENM zu einer gesteigerten Ökotoxizität führt, sollten die ENM in zwei Gruppen eingeteilt werden: (I) ENM, deren Ökotoxizität durch Alterung beeinträchtigt wird und (II) ENM, deren Ökotoxizität konstant bleibt. Bei ENM, von denen angenommen wird, dass deren Ökotoxizität durch einen Alterungsprozess steigt, sollte sowohl das Ausgangsmaterial als auch ein in dem jeweiligen Testmedium gealtertes Produkt untersucht werden. Für ENM, deren Ökotoxizität sich nicht verändert oder abnimmt, wird die Untersuchung von Ausgangsmaterial als ausreichend angesehen.

2.2.2 Spezifischer Aspekt: Alternative Testsysteme als Bestandteil der Teststrategie für die Beurteilung von ENM

Die OECD-Testrichtlinien sowie weitere Richtlinien wurden hinsichtlich ihrer grundsätzlichen Anwendbarkeit auf die Untersuchung von ENM überprüft und die Eignung bestätigt. Da jedoch einige Anpassungen notwendig sind werden die bestehenden OECD-Richtlinien derzeit überarbeitet und neue entworfen.

Neben den standardisierten Testverfahren werden in der Literatur auch alternative Testverfahren für die Untersuchung von ENM beschrieben. Noch ist es nicht offensichtlich, ob diese alternativen Testverfahren im Rahmen einer regulatorischen Beurteilung von ENM Zusatzinformationen liefern und daher in eine Teststrategie integriert werden sollten. Im Rahmen des vorliegenden Projektes wurden alternative Testverfahren im Rahmen einer Literaturrecherche identifiziert (z.B. Verhalten, ernährungsmäßige Effekte, Indikatoren für Sauerstoffstress, Hämatologie, Histologie, Genotoxizität, Cytotoxizität, Neurotoxizität, Immunotoxizität, Bioakkumulation, Biodiversität). Hieraus ergaben sich folgende Schlussfolgerungen:

1. Die konventionellen Endpunkte, die bislang für die Risikobeurteilung herangezogen werden, werden im Hinblick auf den Schutz von Populationen ausgewählt und umfassen Parameter wie Reproduktion, Mortalität und Wachstum. Der Schutz von Individuen steht nicht im Vordergrund. Die alternativen Endpunkte, die in der Literatur beschrieben sind, betreffen in der Regel weniger komplexe Reaktionen (z.B. die Erfassung von spezifischen Enzym- oder Genaktivitäten) mit häufig gesteigerter Sensitivität. Es wird nicht immer offensichtlich, ob ein Effekt, der mit einem sensitiven zusätzlichen alternativen Endpunkt detektiert wurde (z.B. Indikator für Sauerstoffstress), eine Veränderung auf Populationsebene anzeigt, oder ob es sich um einen Kompensationsmechanismus des Organismus handelt. Jeder zusätzliche Endpunkt kann weitere Informationen zur Ökotoxizität von ENM liefern und kann die Beurteilung unterstützen. Für die Aufklärung von

Wirkmechanismen spielen alternative Endpunkte im Rahmen der eine große Rolle und verbessern die Beurteilung der ENM. Im Rahmen der Regulation erscheint der Vorteil gegenüber der derzeitigen Praxis jedoch gering.

2. Einige spezifische Effekte, die sich prinzipiell auch auf Populationsebene auswirken können, werden mit den derzeitigen Testsystemen jedoch nicht erfasst und könnten relevant für die Beurteilung des Gefährdungspotentials von ENM sein.

   a. Immunotoxizität / Gentoxizität
   Die Kenntnisse zur Signifikanz von immunotoxischen und genotoxischen Effekten von ENM in-vitro und auf Populationsebene sollten erweitert werden. Ferner sollten die Ergebnisse mit denen verglichen werden, die im Rahmen von humantoxikologischen Studien gewonnen werden. Damit kann entschieden werden, ob Querschlüsse zwischen Humantoxikologie und Ökotoxikologie ausreichend sind oder diese Parameter zusätzlich in eine Teststrategie zur Beurteilung ökotoxikologischer Effekte von ENM aufgenommen werden sollten.

   b. Bioakkumulation
   Noch liegen keine Informationen vor, welche physikalisch-chemischen Parameter als Indikatoren für eine Akkumulation von ENM herangezogen werden können. Um Informationen zur potentiellen Akkumulation von ENM zu erhalten, schlagen wir eine pragmatische Vorgehensweise vor. So empfehlen wir, die Gehalte der ENM in geeigneten Testorganismen (z.B. terrestrische und aquatische Oligochaeten, Daphnien, Fischembryonen und Pflanzen) aus ökotoxikologischen Studien am Ende der Inkubationszeit zu bestimmen. Deuten die Ergebnisse auf eine Akkumulation hin und werden weitere Informationen gewünscht, können spezifische Studien durchgeführt werden, wobei die Diskussionen im Rahmen des OECD-Expertentreffens (Kühnel & Nickel, 2014) sowie das Vorliegen entsprechender Leitfäden zu ENM zu berücksichtigen sind. Die orientierenden Untersuchungen könnten auch bereits zur Identifikation von geeigneten PC-Parametern beitragen.

   c. Multi-Generationentests
   Es lässt sich nicht ausschließen, dass Effekte deutlicher werden, wenn Mehr-Generationenstudien durchgeführt werden. Auch Wiedererholungsstudien können interessante Informationen liefern. Im Rahmen von regulatorischen Untersuchungen erscheint der Zusatzaufwand jedoch nicht gerechtfertigt. So werden Unsicherheiten bei der Risikobeurteilung durch Bewertungsfaktoren berücksichtigt. Weiterhin gehen wir davon aus, dass die erhöhte Sensitivität von Multi-Generationentests nicht ENM-spezifisch ist, sondern auch auf traditionelle Chemikalien zutrifft, für die die herkömmliche Risikobeurteilung seit vielen Jahren erfolgreich angewendet wurde.

   d. Weitere Testorganismen
   ENM agglomerieren in aquatischen Systemen, so dass aufgrund von Sedimentation erhöhte Konzentrationen auf dem Sediment erwartet werden. Die Organismen *Chironomus riparius* und *Lumbriculus variegatus*, die gemäß der standardisierten Testrichtlinien herangezogen werden, entwickeln sich im Sediment. Es kann nicht ausgeschlossen werden, dass Organismen, die auf

dem Sediment leben oder dieses abgrasen, stärker exponiert werden. Gleiches gilt für Makrophyten, die durch ihre Verzweigungen stärker ENM aus der Wasserphase herausfiltern können. Wir empfehlen, die Sensitivität potentiell geeigneter Organismen (Sedimentorganismen, aquatische Makrophyten) im Vergleich zu den Standardorganismen(*Chironomus riparius, Lumbriculus variegatus, Lemna minor*) zu überprüfen. Basierend auf diesen Ergebnissen kann über die Eignung zusätzlicher Organismen entschieden werden oder ob die traditionellen Organismen für die Untersuchung von ENM ersetzt werden sollten.

e. Verhaltenstests
Bei der Literaturrecherche zeigte sich eine hohe Sensitivität von Verhaltenstests, doch sind die Informationen zur allgemeinen Einsetzbarkeit derartiger Tests für eine Vielzahl von ENM limitiert. Um weitere Informationen bezüglich der Eignung von Verhaltenstests zu erhalten, wurde im experimentellen Teil dieses Projektes der Regenwurm-Vermeidungstest mit *Eisenia fetida* (ISO 17512-1) im Vergleich zum Reproduktionstest (OECD 222) näher betrachtet. Für traditionelle Chemikalien ist bekannt, dass die Sensitivität dieses Tests die des Reproduktionstests erreicht oder übersteigt. Fünf ENM des OECD Sponsorship-Programmes (CeO_2: NM-211, NM-212, ZnO: NM-111, CNT: NM-403, Ag: NM-300K) sowie AgNO3 wurden in beiden Testsystemen untersucht. Die ausgewählten Testsubstanzen unterschieden sich in ihrer Löslichkeit und Stabilität.

Es zeigte sich, dass der Vermeidungstest mit seiner kurzen Testlaufzeit interessante Informationen liefern kann, doch kann eine allgemeine Anwendung als Screeningtest nicht empfohlen werden. In Abhängigkeit der ENM-Eigenschaften können verschiedene Informationen erhalten werden.

- Der Vermeidungstest liefert Informationen zur Ökotoxizität von ionenfreisetzenden ENM und kann für ein Ranking dieser Substanzen genutzt werden. Die Ökotoxizität von ENM kann jedoch mit diesem Test unterschätzt werden, wenn er unmittelbar nach Zugabe schwer löslicher ENM in den Testboden gestartet wird oder die untersuchten ENM durch Alterung toxischer werden.
- Der Vermeidungstest eignet sich, um erste Informationen zum Verhalten von ENM in komplexen Umweltmedien zu erhalten. Noch fehlen die analytischen Methoden, um ENM in derartigen Medien zu charakterisieren. Dabei muss jedoch berücksichtigt werden, dass abnehmende Toxizität aber auch auf reduzierte Bioverfügbarkeit beispielsweise infolge von Sorption zurückgehen kann. Die Ergebnisinterpretation hat daher mit höchster Sorgfalt zu erfolgen.
- Es besteht Forschungsbedarf, um die Diskrepanz zwischen Wirkung und Konzentration bei einigen ENM zu erklären (fehlende Konzentrations-Wirkungs-Beziehungen bei schwer löslichen ENM).

2.3 Beurteilung des Umweltverhaltens

Zur Auswahl geeigneter Parameter, Testdesigns und –methoden für eine Teststrategie zur Unterssuchung des Umweltverhaltens von ENM, umfasste die vorgenommene

Literaturrecherche mit dem Umweltverhalten verknüpfte Begriffe wie „Löslichkeit“ (solubility), „Verteilung“ (partitioning), „Stabilität in der (aquatischen) Umwelt“ (stability in the (aquatic) environment), „Transformation“ (transformation), „Abbau“ (degradation), „Mobilität und Transport in porösen Medien“ (mobility and transport in porous media), „Sorption / Desorption an Boden, Sediment und Schlamm“ (sorption / desorption to soil, sediment, and sludge), „Bioakkumulation“ (bioaccumulation), „PEC-Berechnung“ (PEC assessment), „PEC-Modelle“ (PEC models) und „Risikobewertungsansätze“ (risk assessment approaches).

2.3.1 Generelle Aspekte

Löslichkeit und Verteilung

Aus der Literaturrecherche wird deutlich, dass die Richtlinien zur Löslichkeit (OECD 105, 107, 117) und zum Verteilungsverhalten (OECD 117, 107, 123) leicht modifiziert werden müssen, um auf Kohlenstoff-basierte ENM wie Fullerene und CNT angewandt zu werden. Derartige modifizierte Testrichtlinien können wertvolle Ergebnisse liefern, wenn die ENM vergleichbarer Natur (z.B. Fullerene, CNT) sind und unter vergleichbaren Bedingungen untersucht wurden. In diesem Fall können die Ergebnisse zum Vergleich und Ranking strukturell vergleichbarer ENM herangezogen werden. Die Verfahren sind jedoch nicht geeignet, um die intrinsischen Eigenschaften von ENM zu beschreiben. Darüber hinaus ist es allgemeiner Konsens, dass Richtlinien, die auf Verteilungsprozessen beruhen, für ENM in der Regel ungeeignet sind, da ENM keinen Gleichgewichtsprozessen mit den umgebenden Medien unterliegen. Am vielversprechendsten sind Ansätze, die komplexe Umweltsysteme nachstellen, da sie die komplexe Natur der ENM sowie ihre komplexen Wechselwirkungen mit der Umgebung integrieren.

Stabilität in der (aquatischen) Umwelt

Unter diese Überschrift fallen auch Agglomeration / Aggregation sowie Kolloidstabilität. Verschiedene Arbeiten beschäftigen sich mit dem Einfluss von Umgebungsparametern wie pH-Wert, Ionenstärke und Huminsäuregehalt auf die Stabilität der ENM unter wässrigen Bedingungen. Auch hier gilt, dass weitere Forschung notwendig ist, um die Zusammenhänge korrekt zu erkennen und zu beschreiben. Experimentelle Ansätze, die auf modifizierten Standardverfahren beruhen, sind geeignet, um ENM vergleichend zu untersuchen. Für die Beschreibung ihrer intrinsischen Eigenschaften erweisen sie sich dagegen als ungenügend. Hierfür sollten zumindest Modifikationen wie der Zusatz von natürlichen Huminsäuren aus Flüssen oder Bächen berücksichtigt werden. Noch besser sind Versuchsansätze, bei denen die komplexen Umweltbedingungen nachgestellt werden, wie dies beispielsweise mit komplexen Versuchsaufbauten im Labor (z.B. Laborkläranlagen) oder aquatischen Mesokosmen der Fall ist. Obwohl jedoch die Notwendigkeit komplexer Untersuchungsansätze in der wissenschaftlichen Gemeinschaft unbestritten ist, beschäftigt sich keine der identifizierten Literaturstellen mit Detailfragen.

Mobilität und Transport in porösen Medien, Sorption / Desorption an Boden, Sediment und Schlamm

Die meisten Artikel betreffen Mobilität und Transport in porösen Medien und Böden. Die Vorgänge in wässrigen Systemen werden kaum betrachtet, obwohl Sedimentation in Wasser einen wichtigen Prozess darstellt.

Ein besonderer Aspekt ist in der Regel die Betrachtung der Stabilität der ENM während der Untersuchungen sowie deren Charakterisierung und Quantifizierung, da die Bedeutung von

Größe und Größenverteilung für das Umweltverhalten unumstritten ist. Darüber hinaus sollten weitere Parameter wie Form der ENM, Oberflächeneigenschaften und –beschaffenheit berücksichtigt werden.

Es herrscht Einigkeit darüber, dass das Konzept zur Beschreibung der Sorption, das auf Verteilungskoeffizienten beruht und das entscheidend für die Beschreibung von gelösten Substanzen in Boden ist, auf ENM nicht angewandt werden kann, da ENM in Boden, aber auch in anderen Umweltkompartimenten keinen Gleichgewichtsprozessenunterliegen. Dagegen sind weitere Prozesse wie Dispersion, Aggregation, Deposition und Remobilisierung zu berücksichtigen. Aus den Untersuchungen geht deutlich hervor, dass die meisten experimentellen Versuchsaufstellungen die vorliegende Form des ENM signifikant beeinflussen können, so dass die aus dne Versuchen erhaltenen Ergebnissen nicht unbedingt als repräsentativ für das Umweltverhalten zu sehen sind. So liegt das Boden zu Lösungsmittel-Verhältnis, das in Batch-Experimenten gemäß OECD 106 angewendet wird, deutlich unter dem der Realität, wodurch ein verändertes und nicht den realen Gegebenheiten entsprechendes Aggregations- und Löslichkeitsverhalten der ENM hervorgerufen wird.

Einige Studien beschäftigen sich mit der Modifikation der OECD 106, was jedoch in der Regel aufgrund schwieriger Interpretierbarkeit der Ergebnisse nicht überzeugend gelungen ist. So untersuchen Forouzangohar et al. (2011) beispielsweise die Bedeutung hydrophiler Kräfte für das Sorptionsverhalten von Fullerenen, die in einem wasserdominierten Lösungsmittelgemisch stabilisiert waren. Die Autoren stellen die generelle Gültigkeit von Gleichgewichtsreaktionen und Koc-Modellierungsansätzen zur Beschreibung des Verhaltens von nC60 Partikeln in Boden in Frage, schließen jedoch die Anwendbarkeit bei stärker unpolaren Lösungsmittelgemischen (z.B. > 60 % Ethanol) nicht aus. Kiser et al. (2012) untersuchen, wie von der entsprechenden OPPTS Richtlinie gefordert, die Sorption von ENM an Belebtschlamm, wobei frischer Schlamm zu verwenden ist. Diese Vorgehensweise ist zu modifizieren. Die Methode eignet sich, solang der Einfluss unterschiedlicher Klärschlämme untersucht werden soll. Für die Bestimmung der inhärenten Eigenschaften ENM ist das Verfahren fragwürdig, da das Ergebnis von der Aufarbeitungstechnik beeinflusst wird. Ferner ist das Verhältnis von Feststoff zu Flüssigkeitsvolumen (s. oben) zu berücksichtigen.

Generell kann festgestellt werden, dass Verfahren, die die Umweltbedingungen abbilden zu bevorzugen sind. Dies sind beispielsweise Bodensäulen-Versuche, wie in OECD 312 beschrieben. Darüber hinaus sollten verschiedene analytische Verfahren angewendet werden, um ein umfassendes Bild der ENM-Mobilität in porösen Medien und Böden zu erhalten.

Bioakkumulation in aquatischen Organismen

Nur wenige Literaturstellen befassen sich mit der Anwendbarkeit von Bioakkumulationstests auf ENM. Bei allen Untersuchungen zur Bioakkumulation muss berücksichtigt werden, dass die Aufnahme- und Verteilungsprozesse von ENM kinetisch gesteuert sind.

Bioakkumulation (Boden/Sediment)

Tests wie sie in der OECD 315 und 317 vorgestellt sind, sind generell anwendbar. Der Bioakkumulationsfaktor (BAF) ist ein geeigneter Endpunkt, wobei jedoch noch spezifische Leitlinien zu erarbeiten sind. Flüssigapplikation wird bevorzugt, außer es sind Gegenargumente vorhanden. Entkotete und nicht entkotete Würmer sind zu untersuchen

PEC-Beurteilung, PEC-Modelle

Das Umweltverhalten von ENM wird im Wesentlichen durch Aggregation-, Transformations- und Sedimentationsprozessen geprägt. Diese sind nicht durch Gleichgewichtsreaktionen sondern durch kinetische Reaktionen gesteuert, da ENM kein thermodynamisches Gleichgewicht erreichen, sondern als unstabile Suspensionen in der Umwelt vorliegen. Somit sind konventionelle Verteilungsmodelle, die auf Gleichgewichtsprozessen basieren, wie beispielsweise das Verteilungsmodell von Mackay (Modell zur Beschreibung der Verteilung einer Substanz gemäß den Verteilungsgesetzen der Thermodynamik), nicht anwendbar. Es sind neue Modelle für ENM zu entwickeln, die die komplexen Prozesse in der Umwelt berücksichtigen, um realistische Umweltkonzentrationen zu prognostizieren und die Berechnung eines PEC ermöglichen.

Häufig fehlen vertrauenswürdige Daten, beispielsweise zum Ausmaß der Freisetzung in die Umwelt während des Produktionsprozesses und während der Nutzung. Hier können in begrenzten Umfang Wahrscheinlichkeitsdichtefunktionen weiterhelfen.

Ansätze zur Risikobeurteilung

Nur wenige Literaturstellen, die sich mit der Risikobeurteilung von ENM befassen, wurden ermittelt. Diese umfassen:

- Vergleich der Risikobeurteilung von konventionellen Chemikalien und ENM
- Berücksichtigung von Unsicherheiten und begrenzten Informationen
- Berücksichtigung der Veränderung und Transformation von ENM bei der Risikobeurteilung

Unsicherheiten bezüglich einer möglichen Beeinträchtigung und eines Risikos durch ENM wurden von Adam et al. (2014) diskutiert. Die Autoren kombinierten dazu Lebenszyklusanalyse (LCA) und Ansätze zur Risikobeurteilung. Aufgrund der großen Unsicherheiten hinsichtlich Verhalten und Wirkung von ENM sind probabalistische Ansätze wie das „Bayesian network“ anzuwenden. Nowack et al. (2012) zog die Schlussfolgerung, dass sich die Risikobeurteilung von ENM nicht nur auf das Ausgangsmaterial (pristine ENM) beschränken darf, sondern dass auch Veränderungen und Transformationen in der Umwelt zu berücksichtigen sind.

2.3.2 Umweltverhalten: Generalle Schlussfolgerungen basierend auf der OECD WPMN und der Literaturrecherche

Auf Grund der Literaturrecherche und des OECD Expertentreffens werden folgende Hauptschlussfolgerungen gezogen:

- Die veröffentlichten Ergebnisse sind sorgfältig hinsichtlich Nachvollziehbarkeit und der Eignung des angewendeten Methodenspektrums zu evaluieren. Veröffentlichungen ohne genaue Methodenbeschreibungen sind im Rahmen der Teststrategie nicht zu berücksichtigen.
- Besonders für die Bestimmung der Größe und Größenverteilung ist ein Set von verschiedenen analytischen Verfahren anzuwenden, um umfassende Informationen zu erhalten. Generell gilt, dass die Verwendungen von mehr als einer analytischen Methode zu empfehlen ist, um dem komplexen Verhalten von ENM gerecht zu werden.

- Die meisten Versuchsansätze beeinflussen wahrscheinlich den Zustand der ENM und damit auch das Ergebnis, das damit nicht repräsentativ für das Verhalten unter realen Bedingungen sein muss.
- Es sollten gestufte Test- und Risikobeurteilungsstrategien herangezogen werden, um der komplexen Natur von ENM Rechnung zu tragen. Dies gilt auch dafür, dass ENM in der Umwelt kurzlebig sein können. In diesem Fall, sollte die Risikobeurteilung auf der Grundlage der „freien", konventionellen Chemikalie (z.B. dem freien Metallion oder dem gelösten Wirkstoff in Fall von Nano-Pflanzenschutzmitteln) erfolgen.
- Die Risikocharakterisierung sollte auf Basis von gealtertem ENM und nicht allein auf Basis des Ausgangsmaterials erfolgen, da dies das langfristige Vorkommen der ENM in der Umwelt berücksichtigt.
- Je komplexer die betrachtete Umweltmatrix ist, desto mehr Umweltparameter interagieren mit den ENM, die wiederum selbst komplexer Natur sind. Folglich muss das Testdesign folgende Voraussetzungen erfüllen: Je komplexer das untersuchte Umweltkompartiments ist, desto stärker sollte der experimentelle Versuchsaufbau die realen Umweltbedingungen simulieren.
  Dies bedeutet beispielsweise, dass auf der ersten Stufe Verhalten und Abbau in Wasser vergleichsweise einfach untersucht werden kann, unter Verwendung von „Standardwasser" und indem die Bestimmung der Löslichkeit (neue Testrichtlinie erforderlich), Dispersionsstabilität (neue Testrichtlinie erforderlich) und der Stabilität eines ggf. vorhandenen organischen Coatings (neue oder modifizierte Testrichtlinie erforderlich) erfolgt. Auf einer höheren Stufe können Mikro- oder Mesokosmen geeignete Versuchsansätze darstellen.
  Im Gegensatz hierzu sind Verbleib und Verhalten in Boden bereits auf der ersten Stufen in Form einer Boden-leaching-Studie (OECD 312) durchzuführen, die den vertikalen Transport durch den Boden beinhaltet. Bei Durchführung einer gealterten Boden-leaching-Studie wird bereits möglichen Transformationsprozessen Rechnung getragen.
- Die Entwicklung neuer Testmethoden oder die Interpretation von existierenden Verfahren sollten das jeweilige Schutzziel berücksichtigen.
  So sollte beispielsweise diskutiert werden, ob eine modifizierte OECD Testrichtlinie 106 für die Untersuchung von ENM benötigt wird oder ob sie durch die für diese Fragestellung relevantere komplexe OECD 312 (Leaching in Bodensäule) ersetzt wird. In diesem Fall wäre das Schutzziel Grundwasser. Dies bedeutet, dass der Anteil der ENM, der aus der Säule eluiert wird, die Grundwasserexposition widerspiegelt und auf Sorptionskonstanten im Rahmen Risikobeurteilung für Grundwasser verzichtet wird.
- Lebenszyklus- und Risikobetrachtungen sind zu kombinieren.
- PEC-Berechnungen haben zu berücksichtigen, dass das Umweltverhalten (vorwiegend Aggregations-, Transformations- und Sedimentationsprozesse) kinetisch dominiert ist und Gleichgewichtsreaktionen keine Rolle spielen. Entsprechende Modelle sind daher anzuwenden.
- Ein möglicher Ansatz mit fehlender Information zu Input-Parametern für die PEC-Berechnung umzugehen, ist die Berücksichtigung von Unsicherheiten.

3 Introduction

Aim of the project was the proposal for a test and environmental risk assessment strategy for engineered nanomaterials (ENM) addressing the aspects fate and effect. According to the sponsor – the German Federal Environment Agency - the proposed test strategy should be suitable for regulatory purposes and based on experiences and ideas of IME.

Therefore, first a possible strategy ("IME-test strategy") was outlined. This test strategy comprises a tiered approach such as are commonly used in the context of precautionary environmental risk assessment. Typically, such approaches use rather simplistic assumptions, test methods and models on the first tier to give basic information on substance / product inherent properties, and on the predicted environmental behaviour and risk. Depending on the outcome on the first tier, pre-defined trigger values lead to a "stop" of the procedure in case the predicted environmental behaviour and risk is acceptable. If this is not the case, a second or even a third tier is entered comprising higher sophisticated test systems, which take into account specific environmental conditions or even site-specific situations. Besides risk assessment (RA) related aspects, the IME-strategy also takes into account the basic idea of life-cycle assessment (LCA).

In detail, the IME-test strategy can be described as follows:

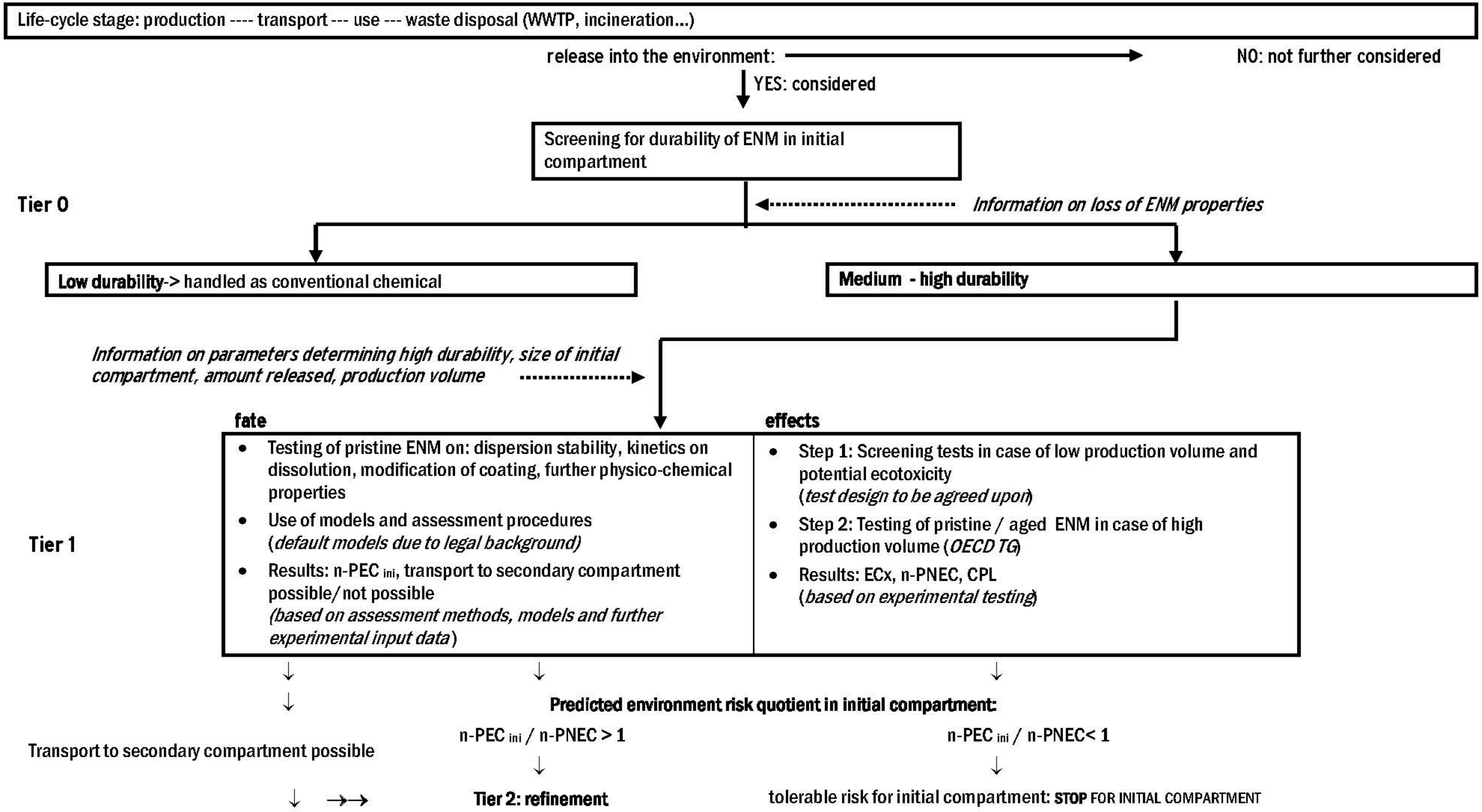
The approach is a life-cycle oriented one, and thus considers all stages along the life of the ENM. In particular these are: production, transport and distribution to the user, use, and waste disposal. Further transport stages might occur, e.g. the transport of the used ENM to an incineration plant. For each single stage it has to be considered whether there is a potential for the ENM to be released into the environment. Furthermore, the initial environmental compartment the ENM is expected to be released into has to be identified. If the release potential is negligible, this particular life-cycle stage needs no further consideration. It has to be discussed and agreed upon elsewhere outside the project what is understood by "negligible" and "non-negligible".

In case the ENM is released into the environment its durability[1] in the initial compartment should be screened (**tier 0**). For that screening any information on the changes or loss of ENM properties is indispensable.Such information should be available (at least to some extend), e.g., from the manufacturer collecting it in the course of product design and development. In case low durability is ascertained, i.e. the ENM rapidly loses its nano-material properties, the formed chemicals can be handled as conventional chemicals. In case medium to high durability is stated, the first tier of the assessment scheme is entered. Any trigger value to differentiate between "high", "medium" and "low" durability has to be discussed and agreed upon elsewhere.

On **tier 1**, both a fate and effect assessment is performed. The assessment results in a predicted environmental concentration in the initial compartment (n-PEC_{ini}) and a predicted no effect concentration (n-PNEC). The prefix "n" is used herein to characterise the PEC and PNEC as concentrations for "**n**anomaterials".

1 On tier 0 the term "durability" is used; it means the presence of the material without changing or loosing the ENM-properties. On the other tiers the term "transformation" is used, which focuses on the individual transformation processes.

Figure 1: Overall test strategy including fate and ecotoxicity.



Tier 2

fate	**effects**
• Testing of pristine <u>and</u> aged ENM on: stability, transformation, transport, mobility (*modified or new OECD TG*) • n-PEC $_{ini\ refined}$ (*based on experimental input data from tier 2, modelling, kinetic models, probabilistic models accounting for uncertainties*) • n-PEC $_{sec\ comp}$ (*based on experimental input data from tier 2, modelling*)	• Higher tier testing / sophisticated tests (*OECD TG, alternative endpoints*) • n-PNEC$_{ini\ refined}$ • n-PNEC$_{sec\ comp}$

↓ ↓

Refined predicted environment risk quotient in initial compartment: n-PEC $_{ini\ refined}$ / n-PNEC$_{refined}$
Predicted environmental risk quotient in secondary compartment: n-PEC $_{sec\ comp}$/n-PNEC$_{sec\ comp}$

n-PEC $_{ini\ refined}$ / n-PNEC $_{refined}$ > 1;
n-PEC $_{sec\ comp}$/n-PNEC $_{sec\ comp}$ > 1
↓
Tier 3: refinement/risk mitigation

n-PEC $_{ini\ refined}$ / n-PNEC $_{refined}$ < 1;
n-PEC $_{sec\ comp}$/n-PNEC $_{sec\ comp}$ < 1
↓
tolerable risk: STOP

The **assessment of n-PEC $_{ini}$** needs the information on experimental data on physical-chemical characteristics as well as preliminary data on environmental behaviour of the ENM, i.e. information on the stability as dispersion or emulsion, stability of the organic coating, modification of the ENM, e.g., by oxidation, dissolution / solubility rate, size, size distribution and shape. It also needs information on the amount of the ENM released as well as on the production volume. It furthermore requires a specification of the size of the initial compartment, e.g., the definition of a local or regional scenario. Finally the definition of default models which is already applied for the exposure assessment of conventional chemicals, plant protection products and biocides is also considered for the derivation of n-PEC $_{ini}$.

The **effect assessment** is based either on screening tests representing the respective initial compartment (in case of low production volume and known nontoxic bulk material; named as "step 1" in the scheme) or on tests using OECD test-guidelines (in case of high production volume; named as "step 2" in the scheme). Thus, the effects testing on tier 1 comprises two different levels of complexity. This is different from the PEC-assessment, where a PEC_{ini} and the transport potential to a secondary compartment are assessed. Trigger values for "low" and "high" production volume have to be discussed and agreed upon. Furthermore, the test design of the screening tests needs mutual consent. NOEC-values or ECx-values are the outcome of any of the experimental testing. Using assessment factors / uncertainty factors as well known from the risk assessment of conventional chemicals a predicted no effect concentration (n-PNEC) can be derived. Besides n-PNEC-values a classification and product labelling (CPL) can be suggested on the basis of the effect concentrations.

Comparably to conventional chemicals, a **risk quotient** (n-PEC $_{ini}$ / n-PNEC) can be formed. In case it is below 1, a tolerable risk for the initial compartment can be assumed. No further sophisticated risk assessment for the initial compartment is needed. In case it is above 1 the risk for the initial compartment might not be negligible and thus, a refinement on tier 2 is needed.

Regardless of the risk quotient, a possible ENM transport to a secondary compartment – e.g. the transport from the aqueous phase to the sediment and transport within the sediment – needs further consideration on tier 2. The transport potential will be assessed on the basis of physico-chemical data, preliminary tests on the environmental behaviour, size, and size distribution rather than on complex fate tests.

The **refined n-PEC-assessment** on **tier 2** comprises two aspects: on the one hand a refinement for the initial compartment (n-PEC $_{ini\ refined}$), and on the other hand an assessment for a second compartment the ENM might be transported into (n-PEC $_{sec\ comp}$). Both need experimental input data and modelling. The experimental fate testing on tier 2 is based on modified or even newly developed test-guidelines. n-$PEC_{ini\ refined}$ and n-PEC $_{sec\ comp}$ are assessed by the use of kinetic models. That accounts for the fact that environmental fate processes of ENM are kinetic processes but no equilibrium processes as they are for conventional chemicals. Consequently, fate models have to be kinetic models. Furthermore, it is advisable – at least on the current state of knowledge – to use probabilistic models in order to account for uncertainties of the model input parameters.

The **refined n-PNEC-assessment** on tier 2 comprises a higher tier testing, i.e. the use of more sophisticated tests such as they are laid down in the OECD guidelines or the use of alternative endpoints. In case of a likely exposure of a secondary compartment appropriate effect tests have to be performed. The testing and the use of assessment factors result in n-$PNEC_{ini\ refined}$ and n-$PNEC_{sec\ comp}$.

Tier 2 yields a refined risk quotient for the initial compartment (n-PEC $_{ini\ refined}$ / n-$PNEC_{refined}$) and – in case of a likely exposure of a secondary compartment – in a risk quotient for that compartment (n-PEC $_{sec\ comp}$ / n-PNEC$_{sec\ comp}$). As on tier 1 the trigger of 1 is used to either "STOP" or to proceed to a further tier. Tier 3 might comprise an additional even more sophisticated test refinement or risk mitigation.

The suggested test strategy has been developed based on the knowledge of national and international discussions, after comparison with proposals presented by the European Commission and by German Federal Authorities. It also takes into account the conclusions made by the OECD WPMN which met in January 2013 (Kühnel & Nickel, 2014).

Furthermore, specific aspects such as alternative testing – which is detailed in the report – were considered by a comprehensive literature search.

Last but not least, one specific topic – namely the appropriateness of the avoidance behaviour test with *Eisenia fetida* (ISO 17512-1) as a screening test on tier 1 – was addressed experimentally.

4 Ecotoxicological test strategy

4.1 General considerations

A test strategy consists of a general procedure including different test systems where chemicals are tested applying standardized test guidelines. The appropriateness of the OECD test guidelines as well as other guidelines for nanomaterials (ENM) have been reviewed (OECD, 2009). It is stated that “the guidance on preparation, delivery, measurement, and metrology is currently insufficient for testing of manufactured nanomaterials”. However, in a further document written as part of the REACH Implementation Projects on Nanomaterials (RIP-oNs) it is explained that the endpoints described and determined in the 31 OECD test guidelines for the determination of potential ecotoxicological effects are adequate and relevant also for ENM (Hankin et al., 2011). Based on the discussions during the OECD expert meeting on ecotoxicology and environmental fate (Kühnel and Nickel, 2014), several member states engaged in the “OECD Working Party on Manufactured Nanomaterials” initiated the update of existing guidelines and the drafting of new ones.

The endpoints described in the standard test guidelines are relevant for the stability of populations. Endpoints such as mortality, growth and reproduction are determined. Besides the application of the standardised test methods, alternative test methods and endpoints are published for the assessment of ENM. Some of the alternative methods cover sensitive endpoints such as enzyme activities and DNA/RNA analyses, but also complex test systems such as multi-generation test systems are suggested. Often they show higher sensitivity than the conventional endpoints mentioned in the test guidelines. So far, it is not obvious whether alternative endpoints provide additional information within the framework of regulation justifying the integration in the test strategy for ENM.

4.2 Alternative endpoints – literature review

4.2.1 Compilation of references

Based on a literature review (performed in 2012; a view additions in 2013) we identified potential alternative endpoints. The aim was to present an overview of key references with a main focus on the photocatalytic ENM TiO_2 and ion releasing ENM Ag. Additionally, the results obtained for further ENM were described with the aim to get information on the specificity of some test parameter.

For the identification of relevant publications the following sources were used:

- Data base of research literature - Science direct
- Data base of research literature – Scopus
- Journal: Nanotoxicology
- Environmental Science of Technology
- Web of knowledge

The following keywords were used:

- “Nanoparticles“ / “Nanomaterials” + “Ecotoxicology“
- “Nano“ + „Ecotoxicology“

- "Titandioxid" + "Ecotoxicology"
- "Silver nanoparticles" + "Ecotoxicology"
- "Nanoparticles"/ "Nanomaterials" + "Soil"
- "Nanoparticles"/ "Nanomaterials" + "Terrestrial "
- "Nanoparticles"/ "Nanomaterials" + "Aquatic"
- "Nanoparticles"/ "Nanomaterials" + "Toxicity"

The alternative endpoints described in the literature were sorted using several generic terms to improve the overview. The following terms were applied:

- Conventional: Parameters used in standardised test guidelines (OECD, ISO) (e.g. mortality, growth, reproduction) or comparable parameters (e.g. growth of bacterioplancton instead of growth of a pure bacterial culture such as of *Ps. putida*)
- Behaviour: behavioural parameters such as avoidance, swimming velocity
- Nutritional performance: parameters dealing with food, such as feeding behavior, feed conversion ratio and feed conversion efficiency
- Indicators for oxygen stress: e.g. TBARS (tribarbituric acid reactive substances), reactive oxygen species, GSH pool, activity of Glutathion-S-transferase, Na+/K+-ATPase, catalase, ascorbate peroxidase, superoxide dismutase, heat shock protein, activity of genes coding for respective enzymes
- Haematology: biochemical parameter determined in the blood
- Histology: microscopic anatomy of cells, tissues and organs
- Genotoxicity: indicators such as DNA strand breaks, micronuclei, comet-assay
- Cytotoxicity: indicators such as neutral red retention assay, lysosomal membrane stability, modifications in single-cell organisms
- Neurotoxicity / immunotoxicity: indicators such as neutrophils, specific biomarkers, activity of respective genes
- Bioaccumulation: determination of the analytical concentration of ENM in organisms or specific organs, tissues and localization of ENM in the animals or plants by microscopy
- Biodiversity: specification of the bacterial community composition using methods such as T-RFLP, Shannon diversity index, substrate richness
- Miscellaneous: indicators which are rather unspecific and not covered by the above listed topics (e.g. tissue electrolyte and trace metal profiles, content of several metals)

In the following table relevant publications are listed.

Sign legend:

(>): Alternative endpoint was affected by the ENM, but the comparison with a conventional endpoint was not possible.

>: Alternative endpoint was affected and seems to be more sensitive than the conventional endpoint.

<: Alternative endpoint was affected and seems to be less sensitive than the conventional endpoint.

=: Alternative endpoint and conventional endpoint showed comparable effects.

-: Alternative endpoint was not affected by the nanoparticles.

+: used only for bioaccumulation; indication for uptake.

Table 1 presents the selected references. The table shows first the references for TiO_2 followed by the references for silver. For both materials the references are sorted according to the test organisms. In some publications further ENM were studied. These results are additionally listed in the table. Some alternative endpoints were only addressed for ENM differing from Ag and TiO_2. These endpoints are listed at the end of the table. Information related to each reference includes the endpoint, organism, test material, a short description of the effects. In the last column some additional remarks such as the sensitivity compared to the conventional endpoints, classification of the information on the alternative endpoints or specific observations are presented.

Table 1: Compilation of the reviewed literature on ecotoxicity of nanomaterials (focus TiO_2 and Ag).

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
			Focus TiO_2			
1.	Behaviour: Total distance moved, time inactive, mean velocity when active and time spent at low, medium and high speeds, activity of aceytlcholinesterase, number of aggressive acts by each fish Indicators for oxygen stress: In brain, gill and kidney - Total glutathione (GSH), activity of Na+/K+-ATPase, concentration of thiobarbituric reactive substances (TBARS) Haematology: Percentage haematocrit, haemoglobin, osmolarity and Na+ and K+ in plasma Histology: General architecture; total number of secondary lamellae with lesions, % of each type of lesion, height of tissue layers in mesencephalon and cerebellum, mean number of melanomacrophages in spleen, liver and kidney Bioaccumulation: Content of Ti in fish (gill, internal organs, brain)	*Oncorhynchus mykiss*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Behaviour: Proportion of time spent swimming at high speed was significantly decreased. Retained competitive abilities when paired with controls in aggressive social encounters. Oxygen stress: Significant increase in oxidative stress defences such as the total glutathione pool, but without loss of Na+K+-ATPase or acetylcholinesterase activities. Haematology: Shift in swimming speed distribution in the TiO_2 ENM-exposed fish was associated with decreased area of red pulp in the spleen, increases in haematocrit and whole blood haemoglobin, all consistent with a compensation for respiratory hypoxia without the accumulation of plasma lactate. Histology: Damage to gill epithelium. Bioaccumulation: 31 (bulk) and 22 fold (nano) increases in the Ti concentrations of gill tissue compared to controls; no measurable increases of Ti in the internal organs including the brain.	Boyle et al. (2012)	Various information about distribution and interactions in fish. Competitive interactions may be transferred to population. Behaviour: (>) Oxygen stress: (>) Haematology: (>) Histology: (>) Bioaccumulation: +

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
2.	Indicators for oxygen stress: Na+/K+-ATPase activity, TBARS and total glutathione. Haematology: Measurement of haematocrit (Hct), haemoglobin concentration (Hb) and calculated mean erythrocyte cell volume (MEV) and mean erythrocyte haemoglobin content (MEH); analysis of plasma ions and osmometry. Histology: Histological observations on the liver, intestine and brain. Miscellaneous: Tissue electrolyte and trace metal profiles - Content of Ti, Cu, Zn, Mn in fish tissue.	*Oncorhynchus mykiss*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No information Oxygen stress: Statistically significant decreases in Na+K+-ATPase activity in the gills and intestine, and a trend of decreasing enzyme activity in the brain. Thiobarbituric acid reactive substances (TBARS) showed exposure concentration-dependent and significant increases (two-fold or more) in the gill, intestine and brain compared to controls. Significant increases in the total glutathione levels in the gills, but depletion of hepatic glutathione compared to controls. Liver cells exposed to TiO_2 ENM showed minor fatty change and lipidosis, and some hepatocytes showed condensed nuclear bodies (apoptotic bodies). Haematology: No effect observed. Histology: Some gill pathologies including oedema and thickening of the lamellae. Miscellaneous: Exposure concentration-dependent changes in tissue Cu and Zn levels were observed, especially in the brain.	Federici et al. (2007)	A couple of different information about the distribution and interactions in fish. Sensitive endpoints; no information on consequences for population. Oxygen stress: (>) Haematology: - Histology: (>) Miscellaneous: (>)

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
3.	Conventional: Growth Nutritional performance: Specific growth rate (SGR), feed conversion ratio (FCR), and feed conversion efficiency Indicators for oxygen stress: Na+/K+-ATPase activity, TBARS and total glutathione Haematology: Measurement of haematocrit (Hct), haemoglobin concentration (Hb) and calculated mean erythrocyte cell volume (MEV) and mean erythrocyte haemoglobin content (MEH); analysis of plasma ions and osmometry Miscellaneous: Whole body electrolyte and trace metal profiles - Content of Ti, Cu, Zn, Mn, Ca, Na and K in fish body	*Oncorhynchus mykiss*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No impact on growth Nutritional performance: No effect observed Oxygen stress: 50% inhibition of Na+K+-ATPase activity in brain (was unaffected in the gills and intestine). Thiobarbituric acid reactive substances (TBARS) showed up to 50% decreases in the gill and intestine. Haematology: No effect observed Miscellaneous: Accumulation occurred in the gill, gut, liver, brain and spleen during dietary TiO_2 exposure. Notably, some of these organs, especially the brain, did not clear Ti after exposure. Brain also showed disturbances to Cu and Zn levels.	Ramsden et al. (2009)	A couple of different information about the distribution and interactions in fish. Comparable low sensitivity for TiO_2. In comparison to endpoint growth: Nutritional performance: - Oxygen stress: > Haematology: - Miscellaneous: >

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
4.	Cytotoxicity: Neutral red retention assay Genotoxicity: Percentage of total DNA in the tail (Comet assay (4h); modified comet assay (24h)); DNA strand breaks using single cell electrophoresis (Comet assay); micronuclei identification and calculation of the Nuclear Division Cytotoxicity Index (NDCI) (Cytogenetic micronucleus assay)	Fish cells of *Oncorhynchus mykiss*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No information Cytotoxicity: Significant reduction in lysosomal integrity over 24 h exposure (NRR assay). Genotoxicity: No effect observed in the absence of UVA irradiation. A significantly increased level of strand breaks was observed in combination with UVA (3 kJ m^{-2}).	Vevers & Jha (2008)	Test with fish cells; exposure not comparable to whole organisms. Cytotoxicity: (>) Genotoxicity: (>)
5.	Conventional: fish embryo toxicity Cytotoxicity (cell culture)	*Onchyrhynchus mykiss* cell line *Danio rerio* embryos	PVP coated Ag-nanomaterials with different shapes and sizes	Conventional: effects on fish embryos depending on ENM shape Cytotoxicity: effects on fish cells depending on ENM shape	George et al. (2012)	Cytotoxicity = ENM plates were more toxic than other shapes Ag ion bioavailability failed to comprehensively explain the high toxicity; reason were crystal defects

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
6.	Conventional: Mortality and development (Zebrafish embryo toxicity assay) Indicators for oxygen stress: Oxidative burst; ROS metabolism; oxidative stress, heat shock proteins, inflammation, antioxidants Immunotoxicity: Neutrophil extracellular traps (NETs), release and degranulation of primary granules (degranulation of primary granules and respiratory burst assay, neutrophil extracellular trap release assay, in vivo neutrophil function assay); leukorgrams (total number of lymphocytes, monocytes, neutrophils and thrombocytes); immune gene expression with quantitative PCR (qPCR) - biomarker Type: normalization genes, metabolic stress, ribosomal proteins, other immune markers.	*Pimephales promelas*, embryos and adults	TiO_2; Average diameter 43 – 86 nm depending on media	Conventional: No effect on fish embryo (Mortality) Oxygen stress: TiO_2 in vitro stimulated oxidative burst and fish exposed to nano-TiO_2 for 48 h in vivo had significantly increased expression of interleukin 11 (inflammation), macrophage stimulating factor 1 (ROS formation), and neutrophil cytosolic factor 2 (Others). Immunotoxicity: TiO_2 in vitro stimulated NET release. Intraperitoneal injection caused a significant decrease in oxidative burst, NETs release and degranulation (21%; 11%; and 30%, decrease, respectively).	Jovanovic et al. (2011a)	In comparison to endpoint mortality: Oxygen stress: > Immunotoxicity: >
7.	Neurotoxicity/immunotoxicity: Changes in the expression of genes involved in cross talk of the nervous and immune systems (Total RNA extraction; microarray hybridization; measuring of aRNA concentration with GeneChip 3‘ IVT Express Kit)	Zebrafish embryo; No guideline	TiO_2 nanopowder, anatase, < 25 nm material size	Conventional: No information Neurotoxicity/immunotoxicity: Exposure caused shifts in gene regulation response patterns. Significant effects on gene regulation were observed on genes involved in circadian rhythm, kinase activity, vesicular transport and immune response.	Jovanovic et al. (2011b)	No information to sensitivity compared to conventional endpoint. Similar response in gene down regulation of embryonic zebrafish exposed to TiO2 and hydroxylated fullerene. Neurotoxicity/immunotoxicity: (>)

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
8.	Conventional: Behaviour, mortality and growth Histology: Histological examinations of gill, liver, heart and brain (exercised and weighed) Bioaccumulation: Concentration of TiO_2 in tissues.	*Danio rerio*	TiO_2, Average material size < 10 nm; BET 218,3 m^2/g	Conventional: Adverse effect, including concentration-dependent and time-dependent inhibition of growth Histology: Increase of the liver weight ratio and of the gill weight ratio of zebrafish. Gills displayed histopathologic change including thickening of oedema and the gill lamellae. Bioaccumulation: TiO_2 ENM were accumulated and distributed in gill, liver, heart as well as brain.	Chen et al. (2011)	Equal sensitivity compared to regular endpoints. In comparison to the endpoint growth: Histology: = Bioaccumulation: +

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
9.	Behaviour: Indicator for reproduction - eggs counted as viable or non-viable Indicators for oxygen stress: Total protein content, Na+/K+-ATPase activity and total glutathione; Haematology: Red and white blood cells counted Histology: analysis of tissues via light microscopy Bioaccumulation: whole body Ti levels Miscellaneous: Whole body electrolyte and trace metal profiles - Content of Cu, Zn, Mn, Ca, Na and K in fish body	*Danio rerio*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Behaviour: At the end of the 14-d exposure adult zebrafish were able to reproduce; however, the cumulative number of viable embryos produced was lower in fish exposed to TiO_2 by the end of the 21-d recovery period Oxygen stress: Total glutathione (GSH) levels in brain, gill and liver tissues were higher in fish exposed to TiO_2 ENM compared to bulk TiO_2 and control fish. No effect on Na+/K+-ATPase activity. Haematology: No change in erythrocyte counts were observed, but there was a two-fold decline in leukocyte counts in all TiO_2 treatment groups relative to time-matched controls. Histology: Gill, liver, brain and gonad tissues showed little evidence of treatment-related morphological change. Bioaccumulation: Whole body Ti concentrations increased significantly in fish exposed to both the 1.0 mg /TiO_2 ENM and bulk TiO_2 compared to controls, but concentrations returned to control levels by the end of the recovery period. Miscellaneous: Whole body electrolyte and trace metal profiles were not affected.	Ramsden et al. (2012)	No information about sensitivity compared to conventional endpoints. A couple of different information about the distribution and interactions in fish. Observation of reproductive stress after a recovery period can be relevant for population. Behaviour: (>) Oxygen stress: (>) Haematology: (>) Histology: - Bioaccumulation: - Miscellaneous: -

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
10.	Indicators for oxygen stress: Stress-activated *p38 MAPK* in protein extracts from haemocyte monolayers; extracellular oxyradical production Cytotoxicity: Lysosomal membrane stability; lysosomal enzyme release; Miscellaneous: nitrite production	*Mytilus galloprovincialis*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No information Oxygen stress: Inflammatory effects of ENM were mediated by rapid activation of the stress-activated *p38 MAPK*. Cytotoxicity: ENM suspensions induced a concentration-dependent lysozyme release, extracellular oxyradical Miscellaneous: ENM suspensions induced a concentration-dependent nitric oxide (NO) production.	Canesi et al. (2010a)	Information on consequences for population is not obvious. Oxygen stress: (>) Cytotoxicity: (>) Miscellaneous: (>)
11.	Indicators for oxygen stress: Enzyme activities (catalase, GSH transferase, protein content) Cytotoxicity: Lysosomal membrane stability in haemocytes, digestive gland and gills Miscellaneous: Lysosomal biomarkers (N-acetyl-ß-hexosaminidase activity; neutral lipid content, lipofuscin content)	*Mytilus galloprovincialis*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No information Oxygen stress: ENM increased the activity of the antioxidant enzyme catalase, and stimulated glutathione transferase (GST). Cytotoxicity: ENM suspensions induced significant lysosomal membrane destabilisation in both the haemocytes and the digestive gland. Miscellaneous: TiO_2 induced lysosomal neutral lipid accumulation.	Canesi et al. (2010b)	Test with hemolymph; no information on consequences for population as exposure not comparable to whole organisms. Oxygen stress: (>) Cytotoxicity: (>) Miscellaneous: (>)

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
12.	Conventional: Mortality Nutritional performance / Behaviour: Feeding rate, leaf consumption in darkness over 24h	*Gammarus fossarum*, No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Adverse sublethal effects of nTiO_2 at low concentrations both in presence and absence of ambient UV-irradiation. In absence of UV-irradiation, however, the effects seemed to be driven by accumulation of nTiO_2 at the bottom of the test vessels. Adverse sublethal and lethal effects on gammarids caused by the combined application of nTiO_2 and ambient UV-irradiation are suggested to be driven by the formation of reactive oxygen species.	Bundschuh et al. (2011)	Feeding activity has high sensitivity. Nutritional performance / Behaviour: >
13.	Conventional: Immobilization and mortality; days to first brood, average offspring in each brood, and total living offspring, adult survival Bioaccumulation: With and without feeding	*Daphnia magna*, (Acute test OECD 202; Chronic toxicity test OECD 211)	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: nTiO_2 exerted minimal toxicity to daphnia within the traditional 48 h exposure time, but caused high toxicity when the exposure time was extended to 72 h. Chronic exposure to nTiO_2 for 21 d, daphnids displayed severe growth retardation and mortality, as well as reproductive defects. Bioaccumulation: Significant amount of nTiO_2 was found accumulated in daphnids. Daphnids displayed difficulty in eliminating nTiO_2 from their body, presenting increased bio-concentration factor (BCF) values	Zhu et al. (2010a)	Bioaccumulation: +

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
14.	Conventional: Mortality and reproduction Genotoxicity: DNA damage (Comet assay)	*Daphnia magna*; *Chironomus riparius* (*D. magna*: Acute test OECD 202; Chronic toxicity test OECD 211; no guideline for tests with *C. riparius*)	TiO_2 material size 7 nm and 20 nm; SiO_2 material size 7 nm and 10 nm; CeO_2 material size 15 nm and 30 nm.	Conventional: No or only small effects; depending on ENM Genotoxicity: TiO_2 nanomaterial did not lead to significant alterations in geno- or ecotoxic parameters of both species. CeO_2 may have genotoxic effects on *D. magna* and *C. riparius*, given that the DNA strand breaks increased in both species when exposed to this nanoparticle. Significant correlation was observed between DNA damage and mortality in the CeO_2-exposed C. riparius, which suggests that CeO_2-induced DNA damage might provoke higher-level consequences. SiO_2 did not seem to affect the DNA integrity; whereas, the mortality of both the SiO_2-exposed *D. magna* and *C. riparius* increased.	Lee et al. (2009)	Genotoxicity: -/>
15.	Bioaccumulation: Biomagnification and bioconcentration	*Daphnia magna*; *Danio rerio*; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No information Bioaccumulation: No bio-magnifications of $nTiO_2$. *D. rerio* could accumulate $nTiO_2$ by aqueous exposure with high bioaccumulation factors (BCFs)	Zhu et al. (2010b)	Bioaccumulation: +
16.	Multi-generation experiments, acute, chronic	*D. magna*	TiO_2 P25 (Evonik), A-100 (Crenox)	Juvenile offspring of adults that were previously exposed to titanium dioxide nanoparticles exhibit a significantly increased sensitivity to titanium dioxide nanoparticles compared with the offspring of unexposed adults. Adults exhibited no differences among treatments in terms of typically assessed endpoints, such as sensitivity, number of offspring, or energy reserves.	Bundschuh et al. (2012)	Information on long-term behaviour.

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
17.	Conventional: Inhibition of cell growth Cytotoxicity: Cell ultrastructure and surface change (with SEM and TEM) Miscellaneous: Photosynthetic activity; content of chlorophyll a, b and carotenoid; Lipid peroxidation - Protein and malondialdehyde concentration Bioaccumulation	*Chlamydomonas reinhardtii*. No guideline	TiO_2 nanopowder; anatase/rutile; average material size 21 nm; BET 35 – 65 m^2/g	Conventional: nano-TiO_2 inhibited cell growth Cytotoxicity: Electron microscopy images indicated that as concentrations of nano-TiO_2 increased, a large number of *C. reinhardtii* cells were noted to be damaged: the number of chloroplasts declined, various other organelles were degraded, plasmolysis occurred. Miscellaneous: nano-TiO_2 inhibited photosynthetic efficiency, but the content of chlorophyll a content in algae did not change, while carotenoid and chlorophyll b contents increased. Malondialdehyde (MDA) content reached maximum values after 8 h exposure and then decreased to a moderately low level at 72 h. Bioaccumulation: TiO_2 nanoparticles were found to be located inside cell wall and membrane.	Chen et al. (2012)	In comparison to cell growth endpoint: Cell structure: = Miscellaneous: =/< Bioaccumulation: +
18.	Conventional: Inhibition of growth and reproduction Nutritional performance: Dynamic energy budget model - To quantify the overall energy budget (food ingested and assimilated) Bioaccumulation: Localization in the animal body with microscopy	*Ceriodaphnia dubia*, 3-brood reproduction test was performed according to EPA Method 1002	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm; Al_2O_3	Conventional: Inhibited the growth *of C. dubia*. Nutritional performance: TiO_2 ENM could disrupt the assimilation and consumption of energy in *C. dubia* dramatically Bioaccumulation: Not observed.	Li et al. (2011)	In comparison to growth endpoint: Nutritional performance: ≥ Bioaccumulation: -

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
19.	Conventional: Inhibition of bacterial cell growth under light and dark conditions	*Escherichia coli* DH5a; *Bacillus subtilis* CB310; No guideline	TiO_2 (66 nm, 950 nm (anatase/rutile), and 44 mm (pure anatase) advertised material size), SiO_2 (14 nm, 930 nm, and 60 mm advertised material size), and ZnO (67 and 820 nm advertised material size) powders	Conventional: Antibacterial activity generally increased from SiO_2 to TiO_2 to ZnO, and *B. subtilis* was most susceptible to their effects. Oxygen stress: The presence of light was a significant factor under most conditions tested, presumably due to its role in promoting generation of reactive oxygen species (ROS). Bacterial growth inhibition was also observed under dark conditions, indicating that undetermined mechanisms additional to photocatalytic ROS production were responsible for toxicity.	Adams et al. (2006)	---
20.	Cytotoxicity: live/dead BacLight kit; light and dark conditions	*Escherichia coli, Áeromonas hydrophila*	TiO_2 with various morphologies	Negligible cytotoxicity in the dark; significant cytotoxicity with illumination; *E.coli* more sensitive than *A. hydrophila*	Tong et al. (2013)	Cytotoxicity: (>)
21.	Indicators for oxygen stress: Formation of ROS Cytotoxicity: Cell membrane integrity – under dark and light conditions	Planktonic and biofilm communities; No guideline	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No information Oxygen stress: Increase of ROS production in the presence of light Cytotoxicity: Microbial membranes were significantly compromised, even under ambient ultraviolet radiation and nano-TiO_2 concentrations predicted for surface waters. Cell membrane damage was more pronounced in free-living cells than in biofilm cells.	Battin et al. (2009)	Cell membrane integrity can be used as indicator for development of populations. Oxygen stress: (>) Cytotoxicity: (>)

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
22.	Conventional: Cell counts and growth rate	Phytoplankton: *Thalassiosira pseudonana, Skeletonema marinoi, Dunaliella tertiolecta, Isochrysis galbana*	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: Inhibition of growth	Miller et al. (2010)	Reference selected as algae species were tested which are not commonly used
23.	Conventional: Soil basal respiration and substrate induced respiration (SIR) Biodiversity: Bacterial community composition with terminal restriction fragment length polymorphism (T-RFLP) analysis	Microbial soil community; No guideline	TiO_2, material size 15-20 nm; 81% anatase, 19% rutile ZnO, material size 20-30 nm, 100% zincite	Conventional: Total soil respiration indicated impacts on overall microbial activity. Nano-TiO_2 and nano-ZnO reduced microbial biomass Biodiversity: Nano-TiO_2 and nano-ZnO reduced diversity (by T-RFLP) and altered the composition of the soil bacterial community.	Ge et al. (2011)	Functional and structural microbial diversity is a sensitive endpoint and changes in the microbial composition reflect effects to population. In comparison to microbial activity endpoint: Biodiversity: +
24.	Behaviour: Ability to rebury in clean sediment Genotoxicity: Percentage DNA in the comet trail in 100 cells (Comet assay); Cytotoxicity: Time taken for 50% of the cells to show signs of due leakage (Neutral red retention assay) Bioaccumulation: TEM observations of the gut	*Arenicola marina*; OECD/ICES A. marine burrowing bioassay	TiO_2 primary material size 23.2 nm; anatase/rutile; specific surface area 46.3 m^2/g SWNT; outer diameter 1-2 nm; length 0.5 – 2.0 µm; mean surface area 420 m^2/g	Conventional: No information Behaviour: Significant decrease in casting rate Genotoxicity/Cytotoxicity: Increase in cellular damage and DNA damage in coelomocytes was measured for nano TiO_2. Bioaccumulation: Coherent anti-stokes Raman scattering microscopy (CARS) located aggregates of TiO_2 of >200 nm within the lumen of the gut and adhered to the outer epithelium of the worms, although no visible uptake of particles into tissues was detected.	Galloway et al. (2010)	Casting rate similar to avoidance may be a first indicator for occurring effects, especially by testing metal and metal oxide nanoparticles. Behaviour: (>) Geno- and Cytotoxicity: (≥) Bioaccumulation: -

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
25.	Indicators for oxygen stress: Activity of superoxide dismutase (SOD), catalase (CAT) Genotoxicity: Tail DNA and Olive tail moment (OTM) for evaluating DNA damage (Comet assay) Bioaccumulation: Content in worms Miscellaneous: malondialdehyde (MDA) and cellulose levels	*Eisenia fetida*; Earthworm acute toxicity test (OECD 207)	TiO_2, diameter 10 - 20 nm; rutile; surface area 120 – 130 m²/g ZnO; diameter 10 – 20 nm; rutile; surface area 111.8 m²/g	Conventional: No information Genotoxicity/Oxygen stress: Response of the antioxidant system combined with DNA damage endpoint (comet assay) indicated significant damage to earthworms. Bioaccumulation: Ti and Zn, especially Zn, were bioaccumulated, and mitochondria were damaged. Miscellaneous: Activity of cellulase was significantly inhibited when organisms were exposed to ZnO ENM.	Hu et al. (2010)	No information about sensitivity compared to conventional endpoints. Oxygen stress: (>) Genotoxicity: (>) Bioaccumulation: + Miscellaneous: (>)
26.	Conventional: Lethality; reproduction; juvenile growth Behaviour: Avoidance test – avoidance net response	*Eisenia fetida*; Earthworm acute toxicity test (OECD 207), Earthworm reproduction test (OECD 222), Avoidance test (ISO 17512-1)	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No significant effect on juvenile survival and growth, adult earthworm survival, cocoon production, cocoon viability, or total number of juveniles hatched from these cocoons. Behaviour: Earthworms avoided artificial soils amended with nano-TiO_2.	McShane et al. (2012)	Existing guideline. Behavioural test. Test can be a suitable screening test. For conventional chemicals often sensitivity comparable to reproduction test. In comparison to conventional endpoints: Behaviour: >
27.	Conventional: Growth, fertility and mortality Indicators for oxygen stress: Stress related gene expression with gene *cyp35a2* (semi-quantitative reverse transcription-polymerase chain reaction)	*Caenorhabditis elegans*; No guideline	TiO_2 material size 7 nm; BET 300.8 m²/g; material size 20 nm; BET 66.6 m²/g CeO_2 material size 15 nm; BET 56.4 m²/g; material size 45 nm; BET 5.3 m²/g	Conventional: Decrease in fertility and survival parameters were observed in the 15 and 45 nm of CeO_2 and in the 7 nm of TiO_2 nanoparticles exposure. Oxygen stress: Increase in the expression of the *cyp35a2* gene. Gene knock-down experiment using RNA interference (RNAi) suggested that physiological level disturbances may be related with the *cyp35a2* gene expression.	Roh et al. (2010)	Increase in gene expression and decrease of fertility and survival shows sensitivity compared to conventional endpoint. In comparison to conventional endpoints: Oxygen stress: =

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
28.	Conventional: Animal mass change and mortality Nutritional performance: Feeding rate, defecation rate; food assimilation efficiency (food consumption) Indicators for oxygen stress: Glutathione-S-transferase and catalase activity	*Porcellio scaber*, No guideline	TiO_2; average material size 15 nm; anatase; surface area 190 – 290 m^2/g	Conventional: Weight change and survival were not affected. Nutritional performance: Feeding rate was not affected up to the highest tested concentration. Food assimilation efficiency was not affected up to the highest tested concentration Oxygen stress: Activities of antioxidant enzymes, such as catalase (CAT) and glutathione-S-transferase (GST), in digestive glands were affected in a dose-independent manner.	Jemec et al. (2008)	Feeding activity has high sensitivity. In comparison to weight and survival endpoints: Nutritional performance: - Oxygen stress: >
29.	Conventional: Mortality, weight change Nutritional performance / Behaviour: Feeding behaviour Indicators for oxygen stress: Degree of lipid peroxidation from MDA amount in digestive glands. Cytotoxicity: Cell membrane stability/integrity	*Porcellio scaber*, No guideline	TiO_2; average material size < 25 nm; anatase; surface area 200 – 220 m^2/g	Conventional: No effects on weight change or mortality. Nutritional performance / Behaviour: No effects on feeding behaviour Oxygen stress: Oxidative stress as evidenced by lipid peroxidation was observed at longer exposure durations and high exposure doses. Cytotoxicity: Nano-TiO_2 destabilized cell membranes, but lipid peroxidation was not detected.	Valant et al. (2012)	In comparison to conventional endpoints: Nutritional performance / Behaviour: - Oxygen stress: > Cytotoxicity: >

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
30.	Conventional: Plant growth (fresh weight of shoots); additional root length Indicators for oxygen stress: Activities of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR); lipid peroxidation; phytochelatins (PC) 2,3,4,5 and glutathione contents in plant extracts Histology: localization of ENM in plants Genotoxicity: Micronucleus frequency and phytochelatins levels Bioaccumulation: Total content in plants Miscellaneous: Protein content; Photosynthesis II maximum quantum yield in leaves	*Vicia faba*; No guidelines except Micronucleus frequency (French standard NF T90-327)	Mineral sunscreen nanocomposites, based on a TiO_2 core (10 nm x 50 nm)	Conventional: No observed effect. Oxygen stress: Oxidative stress biomarkers remained unchanged in shoots while in roots, glutathione reductase activity decreased and ascorbate peroxidase activity decreased. Histology: X-ray fluorescence micro-spectroscopy revealed titanium internalization in superficial root tissues Genotoxicity: No observed effect. Bioaccumulation: ICP-MS measurements revealed high Ti and Al concentrations in roots. Miscellaneous: No observed effect.	Foltete et al. (2011)	In comparison to conventional endpoints: Oxygen stress: > Histology: > Genotoxicity: - Bioaccumulation: + Miscellaneous: -
31.	Conventional: Germination rate, dry biomass Bioaccumulation: Localisation of ENM in plants Miscellaneous: Root elongation; evapotranspiration	*Triticum aestivum, Brassica napus, Arabidopsis thaliana*; According to US-EPA guidelines	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: No significant alteration of germination. Bioaccumulation: TiO_2 ENM are taken up by plants. Miscellaneous: Root elongation and evapotranspiration was not inhibited	Larue et al. (2011)	In comparison to conventional endpoints: Bioaccumulation: + Miscellaneous: - Root elongation is difficult to assess at least in soil.

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
32.	Conventional: Germination, mortality, growth rate; additional root elongation	*Avena sativa, Phaseolus aureus, Sinapis alba*; OECD 208	TiO_2 Aeroxide P25; 25% rutile 75% anatase; mean material size 21 nm	Conventional: Inhibition of growth rate. Miscellaneous: Root elongation was not inhibited.	Hund-Rinke & Klawonn (2012)	In comparison to growth rate endpoint: Miscellaneous: <; Root elongation is difficult to assess at least in soil.
			Focus Silver			
33.	Conventional: Mortality Miscellaneous: Basal metabolic rate (BMR) and critical oxygen tension (Pcrit) – oxygen consumption	*Perca fluviatilis*; No guideline	Silver nanomaterial powder, spherical 30 – 40 nm particles; coated with 0.2% PVP	Conventional: No effect observed. Miscellaneous: Nanosilver had no impact on the BMR. Pcrit was increased.	Bilberg et al. (2010)	In comparison to mortality endpoint: Miscellaneous: >
34.	Conventional: Mortality Indicators for (oxygen) stress: metallothionein, heat shock protein 70 (HSP70), glutathione-S-transferase (GST), p53, cytochrome p450 1A (CYP1A) and transferrin (TF) quantified by measuring mRNA concentrations in liver extracts	*Oryzias latipes*; guidance for fish acute toxicity tests (Dir 92/69/EEC (O.J. L383 A, 1992)) and (EPA712-C-96-118, 1996)	Silver nanomaterial powder; average size 49.6 nm; average surface area 50.7 nm^2; zeta potential -29.9 mV	Conventional: No effect observed. (Oxygen) stress: Ag ENM led to cellular and DNA damage, carcinogenic and oxidative stresses, genes related with metal detoxification/metabolism regulation and radical scavenging action were also induced.	Chae et al. (2009)	Distinguishable toxic fingerprints for AgNO3 and silver nanoparticles. (Oxygen) stress: >

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
35.	Conventional: Mortality Indicators of (oxygen) stress: DNA microarray of 207 stress related genes followed by a quantitative qPCR (in liver), metallothioneins, lipid peroxidation. Genotoxicity: DNA strand breaks Bioaccumulation: Hepatic silver content Miscellaneous: Determination of labile zinc and vitellogenin-like proteins.	*Oncorhynchus mykiss*; No guideline	Silver nanoparticles with a mean diameter of 20nm and zeta potential of -5.5mV	Conventional: No mortality (Oxygen) stress: Significant changes in gene expression for 13% of tested gene targets. Levels of metallothioneins were increased. Lipid peroxidation was significantly induced. Genotoxicity: DNA strand breaks were significantly reduced. DNA break levels were lower with nano-Ag and could not be explained by the presence of ionic Ag. Bioaccumulation: Hepatic Ag content was significantly increased in exposed fish. Miscellaneous: Levels of vitellogenin-like proteins were significantly reduced. Levels of labile zinc were increased.	Gagne et al. (2012)	In comparison to mortality endpoint: Oxygen stress: > Genotoxicity: > Bioaccumulation: + Miscellaneous: >
36.	Conventional: juvenile growth Histology Bioaccumulation Miscellaneous: Microarray analysis (4101 clones) with t-RNA from whole larvae or adult tissue (gill, liver, ovary)	*Cyprinodon variegatus*	Ag nanomaterial: dynamic light scattering - primary peak at 35 nm, secondary peak at about 3 nm (presumably result of particle fragmentation resulting from sonication used in the stock preparation)	Conventional: no impact on juvenile growth Histology: significant thickening of epithelia gill tissue Bioaccumulation: significant increases in tissue burdens in both juvenile and adult fish Miscellaneous – gene expression profile: dramatically altered gene expression profiles; greatest in adult gonads, but no dysfunction at tissue level	Griffitt et al. (2012)	Histology: > Bioaccumulation: + Miscellaneous – gene expression profile: > Modification on gill morphology, but very little evidence of effect on gill transcription profiles

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
37.	Conventional: Development, mortality Bioaccumulation	*Danio rerio*	Ag nanomaterial NM-300K	Conventional: EC50 1.1 mg/kg Neurotoxicity: Whole-mount immunostainings of primary and secondary motor neurons also revealed secondary neurotoxic effects. Bioaccumulation: A TEM analysis confirmed uptake of the Ag-ENM, and the distribution within the embryo suggested absorption across the skin.	Muth-Köhne et al. (2013)	Neurotoxicity: < Bioaccumulation: +
38.	Indicators of oxygen stress: ROS formation Cytotoxicity: Membrane integrity and cellular metabolic activity	Hepatocytes of *Oncorhynchus mykiss*; No guideline	Sodium citrate coated Ag nanoparticles (synthesized by reduction of $AgNO_3$); Ag nanoparticles in stock solution mainly present as single particles with the largest fractions being between 1 nm and 5 nm and 5 nm and 10 nm Au nanoparticles	Conventional: No information Oxygen stress: No effect observed. Cytotoxicity: Ag nanoparticles caused a significant reduction in membrane integrity and cellular metabolic activity in a concentration-dependent manner.	Farkas et al. (2010)	No information on consequences for population and about sensitivity compared to regular endpoints. Oxygen stress: - Cytotoxicity: (>)
39.	Multi-generation experiments, reproduction	*D. magna, D. pulex, D. galeata, No guideline*	Silver nanoparticles, NM-300K	Reproduction: *D. magna* affected by all test concentrations *D. pulex*: no statistically significant effects *D. galeata*: high mortality and hence no reproduction at high test concentrations. *D.magna* – recovery experiment: neonates from pre-exposed parental daphnids did not completely recover during two generations in clean water.	Völker et al. (2013)	Information on long-term behaviour.

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
40.	Conventional: Growth inhibition Cytotoxicity: Bacterial cell wall study (gas chromatography-tandem mass spectrometry, circular dichroism) and muramic acid derivation Bioaccumulation: Study of bacteria with TEM	*Staphylococcus aureus*; No guideline	Silver nanoparticle, size mean diameter 18.34 nm, volume mean diameter 4.10 nm, surface mean diameter 2.26 nm	Conventional: Inhibited bacterial growth. Cytotoxicity: Cell wall damage. Some degree of variation in the a-helix position of the peptide chain was observed. Release of muramic acid (MA) into the medium, which could be attributed to cell wall distraction. Bioaccumulation: Localization of Ag ENM in the bacterial membrane.	Mirzajani et al. (2011)	In comparison to conventional endpoints: Cytotoxicity: ≥ Bioaccumulation: +
41.	Conventional: Bacterial activity response (bacterial production/growth) Biodiversity: Extracellular enzyme hydrolysis activity; extracellular alkaline phosphatase activity; extracellular aminopeptidase activity	Bacterioplancton from nine aquatic habitats; No guideline	Carboxyl-functionalized silver nanoparticles; material size 10 nm	Conventional/Biodiversity: After 1 h of exposure, bacterial production and extracellular alkaline phosphatase activity were significantly reduced in all Ag-ENM-exposed samples. After 48 h, bacterial production recovered by 40 to 250% at low Ag ENM nominal concentrations but remained inhibited at the two highest Ag ENM nominal concentrations.	Das et al. (2012)	Effect caused by $AgNO_3$ was slightly stronger than with nanoparticles. In comparison to bacterial activity endpoint: Biodiversity: ≥
42.	Conventional: Growth inhibition Indicators for oxygen stress / genotoxicity / miscellaneous: Different strains sensitive to oxidative stress (*nth-1, sod-2* and *mev-1*), genotoxins (*xpa-1* and *nth-1*) and metals (mtl-2) Bioaccumulation: Localisation of ENM	*Caenorhabditis elegans*; No guideline	Three silver nanoparticles. Two coated with PVP and material sizes of ~21 nm and ~76 nm and one with citrate coating and a material size of ~7 nm. (Measured by TEM)	Conventional: Growth inhibition was found. Various Indicators: A metallothionein-deficient (*mtl-2*) strain was the only mutant tested that exhibited consistently greater Ag ENM sensitivity than wild-type. Bioaccumulation: Organismal uptake and, in one case, transgenerational transfer of Ag ENM. All tested Ag ENM were internalized (passed cell membranes) in *C. elegans*.	Meyer et al. (2010)	Transgenerational transfer (unlaid egg with developing embryo and Ag ENM) was found for Ag ENM, which would be an information useful for considerations about population impacts. Description of a modified growth assay that permits differentiation between direct growth-inhibitory effects and indirect inhibition mediated by toxicity to the food source. In comparison to the growth endpoint: Bioaccumulation: +

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
43.	Conventional: Survival and reproduction Histology: Interactions with biological surface	*Caenorhabditis elegans*; No guideline	Silver nanoparticles, citrate coated, hydrodynamic diameter 50.6 nm, zeta potential -31.33 mV	Conventional: Survival and reproduction of C. elegans evidenced a clear reduction. Histology: Significant interactions of cAgNMs with the biological surfaces (epidemic edema and burst which may be associated with secondary infections in soil ecosystems).	Kim et al. (2012)	Histology: ≥
44.	Indicators for oxygen stress: Changes in levels of expression of nine stress response genes (coding for: CaM, CAT, PKC, MT, HSP60, HSP70, UBQ, SOD, GST) via RNA extractions and qRT-PCR; oxidative damage of proteins (protein carbonyls, 3-nitrotyrosine, protein-bound)	*Eisenia fetida*	Two silver nanoparticles with PVP coating and particles sizes of 10 nm and 30-50 nm. Zeta potential -49.5 mV (10 nm) and -35.9 mV (30-50 nm)	Conventional: No information. Oxygen stress: Responses varied significantly among days with the highest number of significant changes occurring on day three. Significant increases in the levels of protein carbonyls on day three of the exposure to both ions and Ag ENM indicate that both treatments induced oxidative stress	Tsyusko et al. (2012)	No information on consequences for population It is concluded, that toxicity of silver ions and nanoparticles to earthworms is based on the same mechanisms. Oxygen stress: (>)
45.	Conventional: Mortality, biomass change and reproduction Bioaccumulation: Silver content in worm tissue	*Eisenia fetida*; Earthworm reproduction test (OECD 222)	Silver nanoparticles, material size 15 nm	Conventional: No effect on mortality. Increase in biomass and decrease of reproduction in concentration dependent manner. Bioaccumulation: Increase of the ion content in soil with increasing concentration, while the silver concentration in worms is independent from the concentration (steady state at low concentrations).	Schlich et al. (2013)	In comparison to conventional endpoints: Bioaccumulation: -

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
46.	Conventional: Reproduction Bioaccumulation: Content of silver in worms Behaviour: Avoidance	*Eisenia fetida;* Earthworm reproduction test (OECD 222) Avoidance test (ISO17512-1)	Silver nanoparticles: 30 – 50 nm; coated with polyvinylpyrolidoone (PVP) or oleic acid (OA)	Conventional: significant effects on reproduction at 727.6 mg/kg (OA) and 773.3 mg(kg (PVP) Bioaccumulation: concentration dependent accumulation Avoidance: significant effects on avoidance at 9 mg/kg (OA, PVP)	Shoults-Wilson et al. (2011a) Shoults-Wilson et al. (2011b)	In comparison to conventional endpoints: Bioaccumulation: + Avoidance: +
47.	Conventional: Survival and growth rate Genotoxicity: DNA damage (Comet assay) Bioaccumulation: Content of silver in worms	*Nereis diversicolor*, No guideline	Silver nanoparticles; material size < 100 nm	Conventional: No observed effect. Genotoxicity: Ag was able to cause DNA damage (concentration- and Ag form-related). Significantly greater genotoxicity. Bioaccumulation: N. diversicolor did accumulate sediment-associated Ag.	Cong et al. (2011)	In comparison to conventional endpoints: Genotoxicity: > Bioaccumulation: +
48.	Conventional: Growth inhibition (Viability assay) Miscellaneous: Total microbial carbon; total microbial nitrogen Biodiversity: Community level physiological profiles (CLPP); fatty acid methyl ester (FAME) assays - Shannon diversity index (H); substrate richness; number of substrates used in only one sample (optical density >0.25); peak area richness; number of fatty acids present in only one sample (>1%); community characterization via DNA analysis (DGGE)	Arctic soil microbial community; No guideline	Silver (20 nm), copper (20 nm) and silica (15 nm) nanoparticles; silver microparticles (10 µm)	All - Creating a community toxicity indicator: Revealed that of the three ENM examined, silver ENM could be classified as highly toxic to these arctic consortia. Subsequent culture-based studies confirmed that one of the community-identified plant-associating bacteria, *Bradyrhizobium canariense*, appeared to have a marked sensitivity to silver ENM.	Kumar et al. (2011)	Structural microbial diversity is a sensitive endpoint and changes in the microbial composition reflect effects to population. Biodiversity: ≥

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
49.	Miscellaneous: Total microbial carbon; total microbial nitrogen Biodiversity: Community level physiological profiles (CLPP); fatty acid methyl ester (FAME) assays - Mean optical density (at 590nm); Shannon diversity index (H); substrate richness; number of substrates used in only one sample (optical density >0.25); number of fatty acids present in only one sample (>1%); community characterization via DNA analysis (DGGE)	Arctic soil microbial; No guidelines	Mixture of Silver (20 nm), copper (20 nm) and silica (15 nm) nanoparticles	All: Reduction in the ability to use carbohydrate and amino acid substrates and demonstrated an altered pattern of major fatty acid peaks. Polymerase chain reaction–denaturing gradient gel electrophoresis showed consistent differences in the pattern of predominant rRNA gene sequences.	Kumar et al. (2012)	Structural microbial diversity is a sensitive endpoint and changes in the microbial composition reflect effects to population. Biodiversity: ≥
50.	Conventional: Soil microbial biomass (SIR) and basal respiration Biodiversity: Activity of six enzyme substrates representing major pathways of C, N and P cycling in soil (L-leucine-AMC for leucine-aminopeptidase, MUB-b-D-cellobioside for b-cellobiohydrolase, MUB-phosphate for acid phosphatase, MUB-b-D-glucopyranoside for b-Glucosidase, MUB-N-acetyl-b-D-glucosaminide for chitinase and MUB-b-D-xylopyranoside for xylosidase); metabolic quotient, microbial N	Soil microflora; no guideline	Silver nanoparticles; commercially available nanosilver spray „Nano-Argentum 10“	Conventional: Microbial biomass was significantly decreased with increasing ENM-application rate, while basal respiration was increased in this direction. Biodiversity: In addition, metabolic quotients were increased in the SNP treatments compared to the control. No treatment effects were found for microbial biomass N, fluorimetric enzymes, and the abiotic soil parameters pH and soil organic carbon.	Hänsch & Emmerling (2010)	Functional microbial diversity is a sensitive endpoint and changes in the microbial composition reflect effects to population. In comparison to the conventional endpoints: Biodiversity: -

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
51.	Biodiversity: Activity of six exoenzymes related to nutrient cycle (urease, acid phosphatase, arylsulfatase, b-glucosidase) and the overall microbial activity (dehydrogenase, fluorescein diacetate hydrolase)	Soil exoenzyme; No guideline	Silver nanoparticles, citrate coated, average material diameter 9.9 nm, mean material surface area 318 nm^2/particle, hydrodynamic radius in deionized water 20.08 nm	Conventional: No information Biodiversity: Ag ENM were capable of inhibiting the activities of all the exoenzymes tested. Urease and dehydrogenase activities were significantly related to the presence of Ag ENM.	Shin et al. (2012)	Functional microbial diversity is a sensitive endpoint and changes in the microbial composition reflect effects to population. This study suggested that Ag ENM negatively affect soil exoenzyme activities, with the urease activity especially sensitive to Ag ENM Biodiversity: (>)
52.	Conventional: Germination rate, shot length; additional – Miscellaneous: Root length	*Linum usitatissimum, Lolium perenne, Hordeum vulgare*; No guideline	Two silver nanoparticles with an average material size of 0.6 – 2 nm and 20 nm (average surface area 10 – 40 m^2/g). Zero valent iron nanoparticles	Conventional: Reduction in shoot growth was a more sensitive endpoint than germination percentage. Complete inhibition of germination was observed for nZVI. For Ag, complete inhibition was not achieved. Silver nanoparticles inhibited seed germination at lower concentrations, but showed no clear size-dependant effects, and never completely impeded germination. Miscellaneous: No inhibition observed	El-Temsah & Joner (2012)	Parameter is difficult to assess at least in soil. Miscellaneous: <
53.	Conventional: growth Bioaccumulation / sorption: plant Miscellaneous: total nitrogen, total phosphorus, chlorophyll a,b,c	Macrophyte *Elodea canadensis;* No guideline	Silver nanomaterials and CeO_2 nanomaterials	Conventional: high concentrations resulted in growth inhibition Bioaccumulation / sorption: ENM brought into contact with the plants for a short time are either directly accumulated in plant tissues or strongly sorbed on the plants surface	Xiao (2013)	Bioaccumulation /sorption: +
54.	Miscellaneous; high throughput screening with muliparameter cellular responses including ROS production, intracellular calcium flux, mitochondrial depolarization, and plasma membrane permeability.	A human (lung epithelial) and rat (alveolar macrophage) cell line	Spherical Ag, Au, Pt, As_2O_3, SiO_2, ZnO nanomaterials	Sublethal effects	George et al. (2011)	Miscellaneous: (>)

No.	Endpoint	Organism; Performance according to guideline	Material	Effect	Author / year	Remarks on alternative endpoints
			Further ENM used for discussion			
55.	Conventional: toxicity symptoms Bioaccumulation / sorption	Macrophytes *A. caroliniana, E. densa, M. simulans;* No guideline	Au nanomaterials, 4 and 18 nm	Conventional: No visual toxicity symptoms Au ENM adsorbed to root of each species. Differences in adsorption and absorption rate of Au ENM likely are due to the difference in material sizes and differences among the three plant species.	J. B. Glenn et al. (2012)	Bioaccumulation / sorption: +
56.	Conventional: mortality Bioaccumulation	Aquatic ecosystem model with fish (*Pseudorasbora parva*), snails (*Bellamya pruificata*), macrophytes (*Ceratophyllum demersum*)	Ce nanomaterial	No mortality BAF: macrophyllum > snails > fish Ce ENM readily passed through the water column to the organisms; accumulation abilities varied across different organisms.; organisms showed quick accumulation and elimination abilities of Ce ENM; sediments served as the main and ultimate sink of Ce ENM in the system.	P. Zhang et al. (2012)	Bioaccumulation / sorption: +

4.2.2 Interpretation of results

Table 1 contains a very heterogeneous set of data. Alternative endpoints were identified for fish, aquatic invertebrates, algae and aquatic plants as well as for soil microflora, terrestrial invertebrates, and plants. Besides the tests with whole organisms also cell tests (e.g. with haemocytes) were performed. Test organisms beyond the standard groups commonly used for testing were aquatic macrophytes, mussels, further algal species, Ceriodaphnia (no test organism in OECD TGs), marine worms, woodlice, microorganisms (single species), bacterioplancton and arctic soil microbial communities. A wide spectrum of endpoints was addressed and condensed to generic terms (explanation of generic term, see chapter 4.2.1). Various organisms can contribute to a generic term. For every ENM type applied (TiO_2 and Ag), ENM differing in crystalline structure (e.g. rutile and anatase for TiO_2), size, surface area and coating were used in the tests. Only a few studies are available in which alternative and conventional endpoints were addressed.

The following procedure was applied to derive information from the heterogeneous data collection concerning the suitability of the alternative endpoints used as part of a test strategy applied in regulation: In the first paragraph “General observations” overarching aspects are considered which relate to various generic terms. Subsequently every term is addressed and an evaluation of the presented references provided. Selected references are marked by a number in square brackets which corresponds to the number of the reference in the table.

4.2.2.1 General observations

Sensitivity: conventional vs. alternative endpoints

Many alternative endpoints may indicate effects. However, a comparison with conventional endpoints described in the guidelines such as growth and reproduction is usually missing. In some publications information on mortality is mentioned which is the most insensitive endpoint. Many alternative endpoints should have a high sensitivity compared to the conventional endpoints (e.g. reproduction, growth) as most alternative endpoints are more specific (e.g. determination of specific enzymes / gene activities or behavioural endpoints). This statement is supported for instance by the finding of Jemec et al. (2008) [28] for *Porcellio scaber.* Ecotoxicity of TiO_2 on *Porcellio scaber* was shown by changes in the activity of antioxidant enzymes, such as catalase and glutathione-S transferase, but higher level endpoints (e.g. weight change and survival) were not affected. Further examples are the findings of Chen et al. (2011) [8] for *Danio rerio* (behaviour, mortality, growth versus histology) and Foltete et al. (2011) [30] for *Vicia faber* (growth versus oxygen stress, histology).

Variability of the effect

In complex test organisms such as fish the effect on endpoints can differ depending on the organ used for the investigation. For example, Ramsden et al. (2009) [3] showed that Na^+K^+-ATPase activity was affected by TiO_2 in brain, but unaffected in gills and intestine. Thiobarbituric acid reactive substances showed up to 50 % decreases in the gill and intestine. This makes the assessment rather difficult, as it has to be decided for each effect on the relevance for the affected organ and endpoint.

Gene expression

The generic terms “Indicators for oxygen stress” and “Neurotoxicity/immunotoxicity” include endpoints which are partly related to gene expression.

To improve the correlation between gene expression and organism level endpoints, Roh et al. (2010) [27] studied the effects of CeO_2 and TiO_2 nanoparticles on the soil nematode *Caenorhabditis elegans* using gene expression (*cyp35a2*), growth, fertility and mortality as endpoints. They concluded that the investigated nanoparticles induce toxic effects on *C. elegans* and that *cyp35a2* coding for a stress related protein may reflect negative effects on the worm’s fertility. There are indications that *cyp35a2* is involved in fat storage pathway in *C. elegans* (Ashrafi et al. (2003); Menzel et al. (2007)), but further studies on the mechanism by which ENM affect *cyp35a2* gene expression, fertility and mortality are required to better understand the ecotoxicity of ENM (Roh et al. (2010) [27]) and the early warning potential of gene expression *cyp35a2*.

Two completely different ENM with regard to their chemical composition were tested for their toxicity to fish: a metal oxide (TiO_2) and a carbon based nanomaterial (hydroxylated fullerene) (Jovanovic et al. (2011b) [7]). Although the ENM differed in their physico-chemical properties, they caused shifts in gene regulation response patterns that were similar for downregulated genes. Significant effects were observed on genes involved in circadian rhythm, kinase activity, vesicular transport and immune response. The ENM differed in the shifts for upregulated genes. The function of most of the upregulated genes is still unknown, but the involvement of two upregulated genes in the immune system regulation indicates a possible effect of nanoparticle treatment on immune function.

In a further study with fish the authors found a significant effect on gill morphology, but very little evidence for an effect on gill transcription profile (Griffitt et al. (2012) [36]). This observation limits the suitability of gene expression profiles as indicator for morphological effects.

Based on these findings gene expression is considered to be not yet ready for implementation into a test strategy.

Indirect effects

The findings of Li et al. (2011) [18] showed that the test design applied has to be carefully evaluated. The presence of ENM can deplete food supplies due to interactions between ENM and food stuffs, as by adsorption, and may lead to aggregation and destabilisation of ENM. Even the presence of medium hard water commonly used in the EPA toxicity testing protocols, can induce aggregation and sedimentation of ENM, as well as the depletion of biologically accessible food sources or nutrients. Li et al. (2011) [18] studied the photoactive titanium oxide (TiO_2) and a non-photocatalytic aluminum oxide (Al_2O_3) and both ENM revealed similar toxicity in a 3-brood reproduction assay with *Ceriodaphnia dubia*. Aggregation and interaction among ENM, *C. dubia*, and algal cells, major food source of daphnids, played a significant role on the responses of *C. dubia* to ENM. In this case mainly secondary effects due to the reduction of the food supply by the ENM were observed.

Multigenerational effects

Multigenerational studies with various daphnia species were performed by Bundschuh et al. (2012) [16] and Völker et al. (2013) [38]. Increased toxicity in younger generations depending on the daphnia species was determined. Additionally, neonates of pre-exposed parental daphnids did not completely recover when transferred into clean water. For nematodes a trans-generational transfer of Ag ENM was observed (Meyer et al. (2010) [42]).

Photocatalytic active ENM / influence of lighting

The crystalline structure of nanoparticular TiO_2 constitutes their photocatalytic properties. Anatase is used if photocatalytic properties are intended. In the presence of illumination including the wavelengths inducing the effect, reactive oxygen species or radicals are produced which can affect the organisms. In some publications this aspect is considered in the experimental design. All the experiments have been performed with and without appropriate lighting (e.g. Adams et al. (2006) [19], Battin et al. (2009) [21], Tong et al. (2013) [20]). The test conditions described in the test guidelines usually do not support photocatalytic activity. The publications reveal that the consideration of the relevant wavelength increase toxicity and that photocatalytic active TiO_2 can affect significantly microbial communities under natural levels of UV radiation.

There are indications that illumination can affect the behaviour of ENM, independent of photocatalytic properties of the ENM type. It was demonstrated that simulated sunlight induced the aggregation of Ag ENM, causing particle fusion or self-assembly to form larger structures and aggregates (Su-Juan et al., 2014). The modifications were shown by various methods such as transmission electron microscopy, dynamic light scattering and energy dispersive X-ray spectroscopy. However, ecotoxicological effects were not determined. Therefore, it is unknown whether the modification decreases or increases ecotoxicity of non-photocatalytic active ENM.

Behaviour

Studies on the behaviour of different organisms following the contact with nanoparticles can give a first impression about effects to conventional endpoints. It was shown that already at low TiO_2 concentrations earthworms (*Eisenia fetida*) avoided the contaminated soil, while conventional endpoints such as reproduction were not affected (McShane et al. (2012) [23]). A high sensitivity of the avoidance behaviour (*Eisenia fetida*) compared to reproduction was also shown for Ag (Shoults-Wilson et al. (2011a), Shoults-Wilson et al. (2011b) [46]). A study with the marine worm *Arenicola marina* showed that the presence of TiO_2 lead to a decreased casting rate. In this study an effect on conventional endpoints like mortality or reproduction was not observed (Galloway et al. (2010) [21]). In the aquatic environment the behaviour of *Oncorhynchus mykiss* in presence of TiO_2 was determined. Fishes showed significant changes in their behaviour. The proportion of time spent swimming at high speed was significantly decreased when treated with TiO_2. However, the fishes retained competitive abilities when paired with controls in aggressive social encounters (Boyle et al. (2012) [1]).

Nutritional performance

Studies dealing with food uptake and food utilisation were performed with various organisms such as fish, daphnids, gammarids and woodlouse (Bundschuh et al. (2011) [12], Li et al. (2011) [18], Valant et al. (2012) [29]). In the feeding studies with TiO_2, effects were mainly detected on feeding behaviour. Parameters such as feed conversion ratio and feed conversion efficiency were not affected. However, this can depend on the test substance, the selected test concentrations or on the test design.

Oxidative endpoints

In many studies oxidative endpoints were investigated. Two possible scenarios– a direct and an indirect one - can be distinguished: (I) ENM induce the signal for oxidative stress by a reaction with the organism and (II) ENM induce the formation of reactive oxygen species by surface activity of the ENM (e.g. through photocatalytic activity) and the reactive oxygen affectsthe oxidative endpoints. Both scenarioswith varying degrees can be observed for photocatalytic active ENM. In the studies of Adams et al. (2006) [19] the presence of light was a significant factor under most conditions tested, presumably due to its role in promoting the generation of reactive oxygen species (ROS). However, bacterial growth inhibition was also observed under dark conditions, indicating that further mechanisms additional to photocatalytic ROS production were responsible for toxicity. Difference in toxicity on *B. subtilis* between light and dark test conditions was largest for TiO_2 followed by SiO_2. ZnO caused a comparable ecotoxicity, independent ofthe illumination conditions. For *E. coli* the relationship was different. The difference between dark and light test conditions was largest for ZnO, followed by SiO_2 and TiO_2. This indicates that ecotoxicity of ENM is a very complex reaction. In the example of Adams et al. (2006) [19] toxic effects depend on the interaction of ENM, light conditions and test bacterium. The findings on further toxic mechanisms beyond the photocatalytic induced toxicity are supported by investigations of the effects of a rutile form of TiO_2 and ZnO (primary particle size: 10 – 20 nm) on earthworms (Hu et al. (2010) [25]). Antioxidant enzymes were affected by both nanoparticles. Photocatalytic activity as a reason for the ecotoxicological effect can beexcluded: first, rutile TiO_2 reveals significant lower photocatalytic activity compared to anatase TiO_2 and second, the test was performed in soil and hence photoactivation was prevented.

The complexity of interactions of different ENM was shown in a study of Li et al. (2011) [18] with *C. dubia* described in detail above (paragraph “General considerations, indirect effects”) and in a study on biomarkers of mussels with nano carbon black (NCB), C60 fullerene, nano-TiO_2 and nano-SiO_2 (Canesi et al. (2010b) [11]). The ENM increased the activity of the antioxidant enzyme catalase in the digestive gland, with n-$SiO_2 >$ NCB $\approx$ $TiO_2 > C_{60}$. NCB and TiO_2 additionally stimulated glutathione transferase. The effects differ whether gills or digestive glands were investigated. In brief, TiO_2 caused no effect on gill catalase whereas there was a concentration-dependent effect in the digestive gland. SiO_2 caused an effect on gill catalase at one medium test concentration (1 mg/L) whereas there was a concentration-dependent effect in the digestive gland. The applied TiO_2 nanoparticle is a well-known photocatalytic active substance (P25, Evonik) but it causes lower effects than SiO_2 and NCB. Unfortunately, no information on the illumination during the test wasreported and it remains unknown whether photocatalytic reactions were a reason for the observed effects. Additionally, the average size of the primary particles and the surface area differ between the particles and subsequently it is thus difficult to assess the extent of the observed effects on the antioxidant enzymes.

Haematology

In several fish studies haematological analyses were performed, e.g. Boyle et al. (2012) [1], Federici et al. (2007) [2], Ramsden et al. (2012) [9]. Differences of changes in these parameters in fish following treatment with bulk materials compared to fish exposed to ENM or fish from different ENM treatments indicate a material-type effect.

Histological modifications

Histological modifications were studied in higher test organisms, such as fish. Various organs such as gills, liver, brain, gonads were investigated by e.g. Ramsden et al. (2012) [9]; Chen et al. (2011) [8]; Griffitt et al. (2012) [36]. The sensitivity of the investigated modifications can be higher or lower compared to conventional endpoints depending on the investigated organ.

Neurotoxicity / immunotoxicity

Neurotoxicity and immunotoxicity are specific endpoints which may not be expressed by conventional endpoints. Therefore, additional information on toxicity can be achieved by suchmethods. In the reviewed literature these endpoints were addressed by gene expression and are discussed in the paragraph "General considerations, gene expression". Additionally, immunofluorescence staining was performed (Muth-Köhne et al., 2013). For the tested ENM this method was less sensitive than the conventional tests on fish embryos and was discussed as secondary effect.

Genotoxicity

Similar to neurotoxicity / immunotoxicity, genotoxicity caused by ENM may not be detected by application of conventional endpoints. Lee et al. (2009) [13] studied respective biomarkers in daphnids and chironomids for TiO_2, SiO_2 and CeO_2 and compared the results with the conventional endpoints according to the guidelines (daphnids: mortality, growth, reproduction; chironomids: growth, mortality). The investigated ENM types and sizes behave differently with respect to genotoxicity and ecotoxicity. In particular, the 30 nm CeO_2ENM caused genotoxic effects on daphnids, but no effect on conventional parameter such as mortality, growth and reproduction. Also further authors report genotoxic effects of ENM, such as TiO_2 and Ag on various organisms, e.g. Galloway et al. (2010) [24], Hu et al. (2010) [25], Cong et al. (2011) [47]. In the first two references no conventional endpoints were addressed and hence the sensitivity of genotoxic endpoints could not be compared to ecotoxic endpoints. However, ecotoxicity of Ag ENM on *Nereis diversicolor* was lower compared to genotoxicity (Cong et al. (2011) [47]). From the results it can be concluded that ENM can exhibit genotoxicity and therefore, endpoints on genotoxicity can supplement the test programme on ecotoxicity and hence the assessment of ENM. In this context it has to be considered that this is not a nanospecific problem but that it is relevant for all chemicals. Genotoxicity in environmental organisms is not considered in any regulation.

Cytotoxicity

This parameter covers results of tests with cells of complex organisms such as fish cells, blood cells , e.g. Vevers & Jha (2008) [4], Canesi et al. (2010b) [11], Farkas et al. (2010) [38] and results of single cell organisms, such as algae and bacteria, e.g. Chen et al. (2012) [17], Battin et al. (2009) [21], Mirzajani et al. (2011) [40]. In contrast to the exposure of single cell organisms, exposure of individual cells of complex organisms to ENM is an artificial situation and real bioavailability is not included. Subsequently, cytotoxic effects on cell cultures may differ from effects on organisms. For single cell organisms the difference in sensitivity between conventional test parameter and cytotoxicity is assumed to be small as an individual cell stands for the whole organism. For higher and more complex organisms differences in sensitivity cannot be excluded. George et al. (2011) [54] described a high throughput screening using cell cultures. The experiments were performed with human lung cells and the authors demonstrated that the ranking of ENM based on the in-vitro-assay corresponded to the toxicological outcomes in zebrafish embryos, with the exception of Ag-ENM that was more toxic in embryos than in the cells.

Bioaccumulation

The genericterm "bioaccumulation" is used for (I) studies in which concentrations of ENM in the organisms are reported and for (II) studies in which the uptake and localisation of ENM are assessed via microscopy. Uptake of ENM is observed in various terrestrial and aquatic species. Correlation with effects is not obvious at least for ion releasing substances as internal proteins such as metallothioneins can be protecting (Meyer et al. (2010) [42]). Accumulation differs in the various organs of fish or in various parts of plants such as roots, leaves, indicating differences in bioavailability within the organism, e.g. Boyle et al. (2012) [1], Chen et al. (2011) [8], Larue et al. (2011) [31]. Especially daphnids can display difficulty in eliminating ENM from their body, presenting increased bioconcentration factors (Zhu et al. (2010a) [13]). For nematodes, besides internalisation, also transgenerational transfer of Ag ENM was observed (Meyer et al. (2010) [42]). Macrophytes are characterised by many branches and a rough surface. Nanoparticles applied to the water phase could attach to the leave surface and affect the plants which act as kind of filter in the aquatic system (Xiao (2013) [53]). In an aquatic system model consisting of sediment, water, hornworts as macrophytes, fish and snails, ceria ENM were readily removed from the water column and partitioned between different organisms. Hornwort had the highest bioaccumulation factors (P. Zhang et al. (2012) [56]). For nanoparticular Au it was shown that root uptake was size and species dependent. Electron microscopy revealed that 4- and 18-nm Au ENM adsorbed to the roots of each of the tested species. Root tissue was sectioned, and transmission electron microscopy indicated that 4-nm and 18-nm Au ENM were absorbed by *A. caroliniana*, whereas only 4-nm Au ENMwere absorbed by *M. simulans*. *Egeria densa* did not absorb Au ENM of either size. (J. B. Glenn et al. (2012) [55]). Additionally, macrophytes are suitable organisms to study photocatalytic properties of the nanomaterials due to the combination of illumination, water and plants in the test system.

Biodiversity

Effects on soil biodiversity are described for instance by Ge et al. (2011) [23], Kumar et al. (2011) [48] and Hänsch & Emmerling (2010) [50]. Kumar et al. (2011) [48] observed a shift in the microbial soil community exposed to Ag, Cu, SiO_2 nanoparticles characterized via microbial community level physiological profiles and fatty acid methyl ester analysis. Ge et al. (2011) [23] investigated effects on biodiversity and additionally on DNA content, microbial biomass and basal respiration. Nanoparticular TiO_2 and ZnO affected microbial biomass and the composition of the bacterial community (structural biodiversity determined via DNA-patterns), but not basal respiration. This observation can be explained by a change in the microbial community: sensitive bacteria vanished and resistant ones became active. This explanation corresponds with the observed differences in the microbial community characterised via PCR amplification using universal bacterial primers. Microbial biomass, determined as substrate induced respiration (SIR), recovered during the incubation period of 60 days whereas this trend was not observed for the amount of extracted DNA. The authors assume that besides microbial DNA of living microorganisms also DNA of dead organisms or extracellular DNA were extracted and the results therefore reflect a time-integrated effect. However, SIR is a physiological assay that responds only to living cells. This explanation seems to be illogical. If the DNA of living and lysed dead cells is extracted, the differences between treated and untreated samples must be smaller resulting in less obvious effects. The authors excluded a reduction in the extraction efficiency over time and hence the discrepancy remains unclear. Hänsch & Emmerling (2010) [50] investigated Ag ENM and observed a decreased microbial biomass (expressed as microbial C), an increase in microbial basal respiration, but no effect on microbial biomass N and various enzymes representing major pathways of C, N, and P-cycling. The latter are used as indicator for functional biodiversity. Ge et al. (2011) [23] and Hänsch & Emmerling (2010) [50] reported a decreased microbial biomass by ENM and an effect on structural microbial diversity (Ge et al., 2011), but no effect on functional biodiversity (Hänsch &

Emmerling, 2010). Both parameters on microbial diversity provide different, complementary information and are required for a comprehensive assessment of aENM impact.

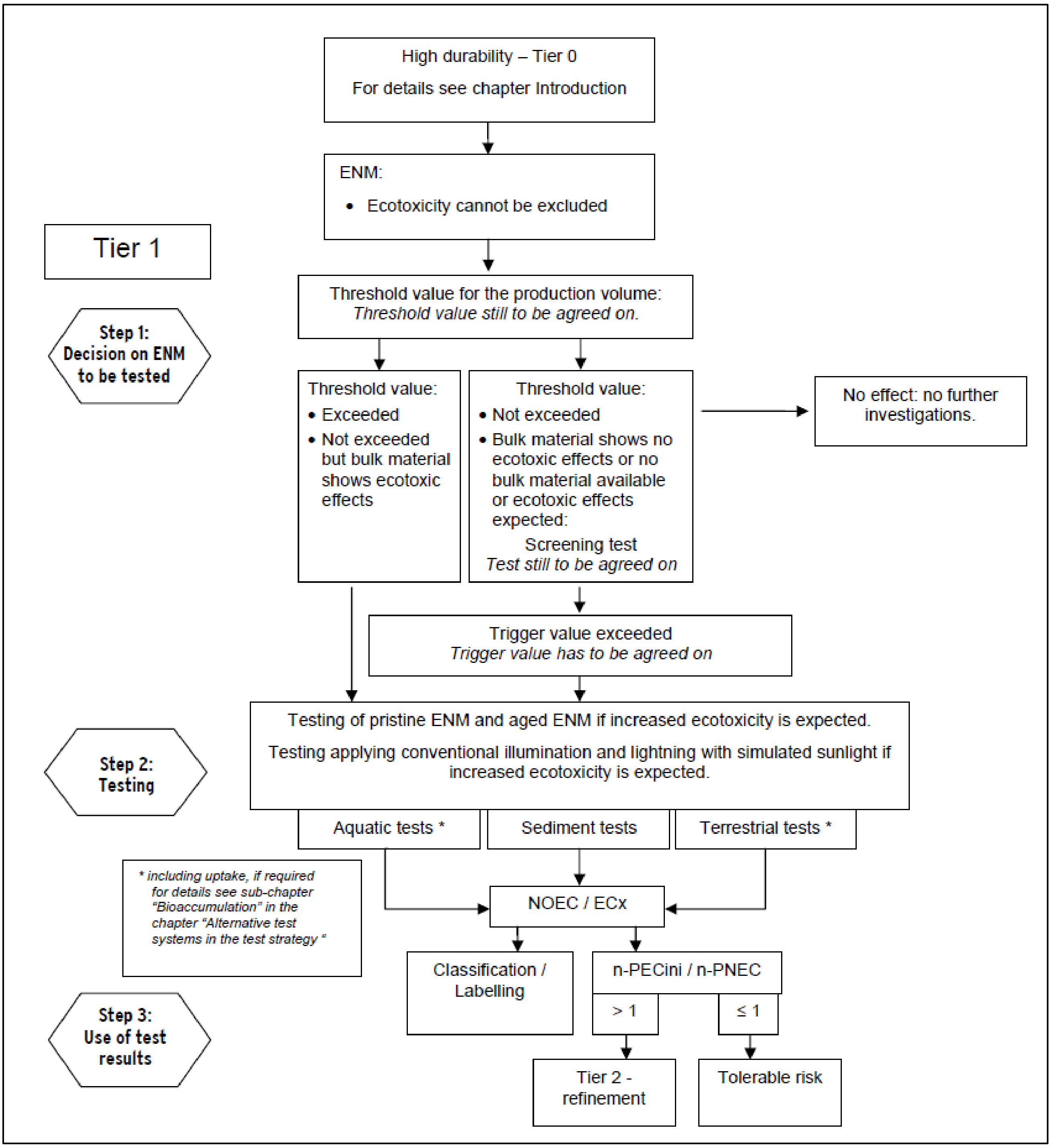
Miscellanteous indicators

The indicators listed under this headline, such as tissue electrolyte, trace metal profiles, oxygen consumption and vitellogenin-like proteins (fish), nitrite production and lysosomal biomarkers (mussel), photosynthetic parameter (algae), root elongation and evapotranspiration (plants), numerous microbial enzymes showed comparable sensitivity as the parameter listed above. They could be affected or not affected and a material-type effect can be concluded. None of the parameters seems to provide outstanding information but every parameter provides additional information.

4.3 Test strategy for ecotoxicological assessment

The current regulations differentiate between "general" chemicals and chemicals with active agent properties such as pesticides and biocides. The ecotoxicological test systems applied for the assessment are the same, but the information requirements within the particular regulation differ. ENM are a very heterogeneous group of substances. Some ENM, such as CNT, are used due to their general properties, e.g. contribution to high stability and flexibility of products. In this case they are comparable to "general" chemicals. A second group consists of ENM which are used because of their active properties. An example is Ag with antimicrobial activity. Furthermore, some ENM, such as TiO_2, belong to both groups. The crystalline form anatase is used because of its high photocatalytic properties, e.g. for "easy to clean" surfaces, whereas the crystalline form rutile is used due to its UV-absorbing properties. Therefore, rutile is rather a "general" chemical, whereas anatase can be classified as chemical showing active agent properties. In the basic test strategy described below such differences are not considered. Figure 2 presents schematically the proposed procedure for the testing of ENM. It includes elements and procedures already used in the assessment of chemicals, products and active ingredients but also further elements to consider specific features of ENM. Differences between the ENM and their intended use might become relevant in the risk assessment which is influenced by regulatory and political aspects. Therefore, some elements of the presented test strategy may not be relevant if the ENM are covered by specific legislations such as biocides and pesticides. However, these aspects are not in the focus of this project. The basic test strategy comprises three phases: (I) decision on the ENM to be tested (II) testing and (III) use of test results.

Figure 2: Basic test strategy for the ecotoxicological testing of ENM (Tier 1).



ENM to be tested

The categorization of ENM is currently in discussion. A category concerning environmental effects contains ENM with comparable ecotoxicity. The attributed PC-parameter or PC-parameter combinations have to be identified. We propose that ENM with PC-parameters indicating no ecotoxicity in any of the environmental compartments (water, sediment, soil) without any doubt do not have to be tested. Furthermore, testing is not required for ENM for which a direct or indirect exposure of the environment can be excluded. ENM which may be toxic and whose production volume exceeds a threshold value as well as the nano-form of ecotoxic bulk material and NM with expected active properties (independent of the production volume) should be tested. The threshold value still needsto be defined in expert groups or committees. This threshold value is a political and strategic decision. Obvious scientific criteria do not exist. The threshold value should consider environmental and economic interests. There should be one threshold value for all ENM. A further differentiation according to production volumes triggering the selection of ecotoxicological tests to be performed is not recommended. This differs from the procedure according to REACH where the production volume triggers the tests which have to be performed. Chemicals with a comparable low production volume have to be investigated just in aquatic tests, whereas chemicals with a high production volume have to be tested in aquatic, terrestrial and sediment tests.Knowledge on the sensitivity of ecotoxicological effects with ENM is still limited and exposure to particles in soil compared to water can differ from exposure to conventional chemicals in these compartments. Therefore, we cannot exclude that ecotoxicity of several ENM in soil exceeds ecotoxicity in water. To ensure that toxic effects are detected we recommend the consideration of the three compartment soil, water, sediment and to define a threshold value that is based on one, rather low production volume.Small production volumes below the threshold value may be of less relevance with respect to environmental exposure and thus environmental damage. However, to detect highly toxic ENM with a small production volume and which are not obviously toxic due their PC-properties, we recommend the performance of a screening test for such ENM. If there is a significant toxicity further testing is required comparable to the testing of ENM with production volumes above the threshold value.

If ENM are covered byspecific legislations where production volumesare not considered (e.g. ENM used as biocides or pesticides), the aspect “production volume”mustbeneglected in the presented test strategy.These ENM should be tested according to the requirements of the respective legislation, but should additionally consider the nanospecific aspects proposed within this test strategy (e.g. selection of tests: chronic tests instead of acute tests).

Testing

Screening test

The screening test and the interpretation of the results still have to be agreed on. The screening test should beused as a kind of analytical device. Therefore, the test need to have a high sensitivity, the indicator function concerning effects on populations is less important. Criteria for such tests should be (I) easy performance, (II) short test duration, (III) sensitivity comparable to sensitivity of the standardised endpoints to avoid too many ”false” positive or “false” negative results. In several publications high throughput assays are described, e.g. Tong et al. (2013), George et al. (2011). Besides testing also modelling might be a useful alternative. By the use ofmodels toxic properties of ENM such as oxidative stress potential of oxide ENM may be predicted (Burello & Worth (2011), H. Zhang et al. (2012)). The suitability of such methods and procedures for the initial examination has to be investigated and the most reliable procedures need to be further developed. If the results indicate significant toxicity, the test program applicable to those ENM whose production volumes exceed the threshold value (see below) has to be performed.

Test systems for ENMwith production volumes exceeding the threshold value or with ecotoxic bulk material or ENM with expected active properties

So far, there are considerable knowledge gapswith regard tothe sensitivity of aquatic tests in comparison to terrestrial tests. It cannot be excluded that terrestrial tests are of comparable sensitivity or even more sensitive than aquatic tests as exposure can differ. In aquatic tests the exposure concentrations and availability can decrease due to agglomeration and – depending on the test conditions - sedimentation. Sedimentation will result in increased exposure for sediment organisms. In contrast, in soil theexposure of the organisms is comparatively stable. Additional agglomeration during the test period is not expected. Therefore, we recommend a test program which includes equivalently the three compartments surface water, sediment, soil. We assume that ENM will preferentially enter the sediment compartment via the water phase. Therefore, we recommend the sediment test performed using spiked water instead of spiked sediment to simulate realistic exposure. Degradability of most ENM is limited due to their inorganic condition and long-term exposure is expected. Therefore, we prefer chronic tests (if available) instead of acute tests. Additionally we recommend tests with organisms which cover the different trophic layers equivalently. We suggest following test program for ENM:

b) Aquatic tests
   - Daphnids: OECD TG 211 “*Daphnia magna* Reproduction Test”
   - Algae: OECD TG 201 “Freshwater Alga and Cyanobacteria, Growth Inhibition Test”
   - Fish: OECD TG 210 “Fish, Early-life Stage Toxicity Test”

c) Sediment test
   - Chironomids: OECD 219 “Sediment-Water Chironomid Toxicity Using Spiked Water“ or Lumbriculus OECD TG 225 “Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment” (so far, a TG for the Lumbriculus test using spiked water is not available)

d) Terrestrial tests
   - Microflora: OECD TG 216 “Soil Microorganisms: Nitrogen Transformation Test”
   - Earthworms: OECD TG 222 “Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*)”
   - Plants: OECD TG 208 - Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test

Due to low sensitivity of the microbial test on substrate induced respiration (OECD TG 217) this test is considered to be of lower relevance and not recommended for the testing program. The test on nitrification is usually more sensitive. However, for ion releasing ENM, the test has to be slightly modified to keep the high sensitivity. Released ions tend to sorb to the additional organic nitrogen source thus reducing their bioavailability and we instead recommend the use of an inorganic nitrogen source or a test on potential ammonium oxidation according to ISO Guideline 15685 (2004) (Hund-Rinke, submitted).

So far, exposure of organisms in aquatic systems is discussed. ENM tend to agglomerate and sediment resulting in a low exposure of the test organisms. The addition of stabilizers to achieve a stable suspension is considered to be less suitable. Within the scope of the EU-project MARINA, work package 10, the impact of natural dispersants such as humic acids and natural organic matter on toxicity of various ENM in the standardised tests with fish, daphnids and algae has beeninvestigated. Due to the observed reduction in toxicity, the use of stabilizers is not recommended. Comparable results were observed applying the chemical dispersant “sodium hexametaphosphate” and Tween 80 (Hund-Rinke and Schlich, not published). Maximum exposure achieved by mechanical treatment such as stirring, shaking and turbulence via ventilation seems to be a better alternative. The vessels used for tests onalgae are shaken and no further treatment is required. For the test with fish and daphnids reduced sedimentation by turbulence via ventilation has beeninvestigated in the EU-project Marina. A suitable procedure must be applicable to many different ENM. So far, results are not available. If it will not be possible to avoid excessive sedimentation, the test strategy has to be modified. In this case, terrestrial and sediment tests gain increased importance. For the tests with sediment organisms spiking of water or of sediment is described in the test guidelines. In the case of water spiking we recommend the acceptance of sedimentation to increase exposure of the test organisms.

In some tests a plateau with a maximum effect below 100 % is observed instead of concentration-effect relationships with a maximum of 100 % effect (Hund-Rinke et al., 2012; Scott-Fordsmand et al., 2008). Therefore, we recommend the investigation of several concentrations per test instead of limit-tests with only the maximum test concentration.

Test approach

The test conditions described in the test guidelines usually do not support photocatalytic activity. Simulated sunlight can increase ecotoxicity of photocatalytic active ENM and possibly also of further ENM types.

Recommendation:

We recommend aquatic tests to be performed according to the guidelines but also with simulated sunlight for photocatalytic ENM. The tests with conventional lighting are recommended to link the results to results obtained by applying the current test guidelines. The most sensitive result, independent of the illumination conditions, should be used for the characterisation and assessment of ENM. In addition the knowledge on the effect of illumination on ecotoxicity of non-photocatalytic active ENM has to be improved.

Use of test results

The test results can be used to describe the *intrinsic*propertiesof*ENM*. Additionally, *classification and labelling* as well as an initial environmental risk assessment can be performed. For eachpurpose only the relevanttest results needto be used. With respect to ecotoxicity, classification and labelling oftraditional chemicals is based on results of aquatic tests. The same procedure can be applied for ENM if it is shown that the aquatic compartment is mostly affected. Currently no literature review comparing the results of terrestrial and aquatic studies on the ecotoxicity of ENM has been performed and it is unknown which is the most endangered environmental compartment which has to be addressed by labels. In addition to that, also the performance of the aquatic tests needs to be evaluated. It is still under discussion whether homogenous and stable test dispersions of ENM using stabilizers have to be used or whether mechanical distribution or– if mechanical distribution reveals to be unsuitable due to a high sensitivity of the test organism (e.g. daphnids) - sedimentation is accepted. Stable test dispersions of conventional chemicals are demanded in the test guidelines. However, addition of stabilizers, even of NOM, can reduce the ecotoxicity of the ENM. To come to a decision on classification and labelling the following approach is proposed:

1. Decision on the procedure to be used for aquatic tests.
   a) Comparatively stable suspensions using stabilizers or
   b) Mechanical distribution
   c) Accepted sedimentation if neither stabilizers nor mechanical distribution are feasible.

   The decision depends on

   a) Practical feasibility of maximum contact without negative impact on test organisms, esp. daphnids, suitability for a wide spectrum of ENM and
   b) Impact of stabilizers on ecotoxicity.
2. Comparison of terrestrial ecotoxicity and aquatic ecotoxicity using the selected approach (see topic 1).
3. Classification and labelling for the most endangered compartment.

For *risk assessment* n-PNEC values are required (prefix "n" used to characterise the PNEC for nanomaterials). They are calculated using appropriate effect data and assessment factors. There are no indications that assessment factors differing from the existing ones are needed for ENM. The n-PNEC values have to be compared with environmental concentrations. However, so far, it is difficult, if not impossible, to characterise ENM in solid matrices such as soil and sediment. Characterisation is limited to the total concentration of the chemical substance, regardless of its particulate nature. Modifications such as destruction of a coating, modification of the crystal structure or size of the ENM by release of ions cannot be detected with acceptable workload. There is a controversial discussion whether only the concentration in the test medium of ions released from ion releasing ENM are responsible for the observed effects or whether additionally particle effects have to be considered. Gomes et al. (2012) concluded in their study on the effects of nanoparticular Cu versus Cu-salt to *Enchytraeus albidus* that effects were probably caused by the ENM themselves and not by released ions. This conclusion was supported by the research of Amorim & Scott-Fordsmand (2012), who observed ENM specific effects of nanoparticular Cu on survival, reproduction and avoidance of *Enchytraeus albidus* exposed to nanoparticular Cu and Cu-salt. In the study of Shin et al. (2012) it was shown, that Ag ENM inhibited the activities of soil exoenzymes while silver ions dissolved from the Ag ENM caused no significant effect, indicating that the adverse effects were caused by the Ag ENM themselves. Roh et al. (2010) showed for *C. elegans* that Ag ENM were slightly more toxic than Ag ions in terms of reproduction potential, and also it appeared that different mechanisms exerted toxicity. Meyer et al. (2010) discussed that ions released from particles in the organisms are relevant for effects and the results of Schlich et al. (2013) strengthened the assumption that toxicity on earthworms caused by Ag ENM is closely related to the content of released Ag^+ measured in the soil pore water. In the latter publication, differences in EC_{50}- values between nanoparticular Ag and silver nitrate were small. DGT measurements revealed no differences between the free Ag^+ content of soils spiked with nanoparticular Ag and silver nitrate. As modifications of the ENM are difficult to determine and the role of ion toxicity for ion releasing ENM is not yet clarified, we recommend the definition of a n-PECini. The PEC-values, specific for ENM and hence marked by the “n-”, refer to the concentration of the pristine ENM, independent on further modifications. With increasing state of knowledge on the influence of modifications or with improved methods concerning characterisation of ENM, the term “n-PECini” can be adapted. We propose to use the same approach for the aquatic compartment to achieve comparability.

The comparison of n-PNEC with n-PEC values implies that the toxicity of substances remains stable. So far, the knowledge concerning the effect of aging on toxicity of ENM is limited. In this context, the term “aging” comprises all modifications/transformations of aENM including the release of ions. It cannot be excluded that toxicity decreases or increases upon aging. Pereira et al. (2011) showed that toxicity for most of the investigated five anorganic ENM decreases with time. However, toxicity increases significantly for Fe/Co magnetic fluid by a factor of about 100 between sampling dates of soil 2 h and after 30 d after spiking. They suggest that aging may have contributed for degrading the organic shell of these ENM. Besides the degradation or transformation of a protecting coating, it cannot be excluded that for ion releasing ENM the size of the ENM decreases by the release of ions followed by an increase in surface area resulting in an increased release ofions. Hence, toxicity could principally increase over time. However, this assumption still needsto be proven and therefore, we recommend stability of ENM assumed unless further information on aging is available. By this a quotient of n-PECini/n-PNEC< 1 reflects a tolerable risk, whereas refinement (Tier 2) is required at a value of ≥ 1. This refinement can cover an improved n $PEC_{ini\ refined}$ instead of the n-PECini considering fate-aspects such as transport in further compartments (n-$PNEC_{sec\ compartment}$), sedimentation and sorption followed by a modified bioavailability. Furthermore, the n-PNEC could be refined by further chronic tests instead of acute tests (e.g. chronic test with plants), by tests resulting in species sensitivity distributions or by more realistic exposure scenarios such as aquatic mesocosm. By this, the assessment factors are reduced.

Recommendation:

For the interpretation of the results exposure should be detailedas much as possible. This includes parameters such as the zeta-potential in the aquatic test medium, description of potential sedimentation and concentration of the ENM in the water phase of aquatic tests. For tests including solid media (soil, sediment) methods to describe the exposure in these media are still missing. At least the test concentration should be verified. If methods on exposure characterisation will be available they can be included in the characterisation of the ENM.

If the knowledge gained by further experiments shows that aging increases ecotoxicity, we recommend the categorization of ENM in (I) materials affected by aging and (II) ENM which are not affected. ENM should be tested twice if it is assumed that ecotoxicity increases by an aging process. We recommend the testing of the pristine ENM as well as of the material aged in the test medium. Testing of pristine ENM is considered to be sufficient if ecotoxicity is expected to decrease or not to be affected by aging.

Metric

In the context with the test strategy, the metric used for the presentation of the toxicity has to be discussed. Size/surface area of the ENM (Roh et al. (2010) [27]) and material properties, (Hu et al. (2010) [25], George et al. (2012) [5], Tong et al. (2013) [20]) affect the toxicity. The effects of conventional chemicals are presented as mass per kg or per litre. For ENM the surface area of the primary nanoparticles and the particle number are further options. Three examples illustrate the consequences using the metrics “mass” and "surface area" (Table 2). The metric "particle number" is not presented separately. The basic information provided by the result based on "particle number" compared to results based on "mass" is covered by the example of the metric "surface area". Two fictive ENM differing in the surface area and with fictive ecotoxicity on mass basis are used for the demonstration. The ecotoxicity on basis of surface area is calculated using the fictive values for surface area and for ecotoxicity on mass basis. The ecotoxicity of both ENM is comparable on mass basis. If they differ in size/surface a clear difference becomes obvious when the surface area is used as basis for the ecotoxicity calculation (example 1). In example 2 the ecotoxicity of both ENM differs on mass basis and the difference becomes even more obvious on basis of surface area. Example 3 illustrates that one ENM can be highly toxic on mass basis but less toxic compared to a second ENM on surface area.

Table 2: Differences in the ecotoxicity of two nanomaterials depending on the used basis (mass vs. surface/particle number).

Nanomaterial [ENM]	Surface [m²/g]	ECx [mg/kg]	ECx [m²/kg]	NM1/NM2 basis: ECx [mg/kg]	NM1/NM2 basis: ECx [m²/kg]
Example 1: different types of nanomaterials, e.g. TiO_2 and Ag					
1	300	50	15	1	30
2	10	50	0.5		
Example 2: different types of nanomaterials, e.g. TiO_2 and Ag					
1	300	500	150	10	300
2	10	50	0.5		
Example 3: the same type of nanomaterial					
1	300	50	15	0.1	3
2	10	500	5		

According to our point of view, there is no clear preference for one option, but the metric to be preferred depend on the situation and the problem. Different cases can be distinguished. Mass seems to be the suitable metric, e.g. in the case of an accident or for the decision of the most profitable ENM. In the case of an accident during the transport, the head of operations has to decide which protective measures have to be initiated. The acute measures are triggered by the amount of the spilled material and the referring toxicity. Also for the decision of the most profitable ENM in a product the mass is the essential parameter as the costs for an ENM are based on the mass basis. Surface area can be an essential parameter in a comprehensive risk assessment usually performed by an expert. Interactions between the surface of the ENM, soil/water constituents and organisms have to be considered in fate and exposure. However, it has to be considered that "the" surface area does not exist. The method and applied absorbent specify the pore sizes which are included and the surface area. An ecotoxicological result (e.g. ECx) and a surface area can only be linked to each other if there is a direct relationship between both, i.e. the specified surface area corresponds to the surface area responsible for the effect.

Recommendation:

Due to the case-dependent requirements, we recommend results presented on the metric "mass". In addition, the surface area, the particle number, the particle size distribution of the ENM and the methods used for the determination should be mentioned. By this, the whole information is available and the effect can be expressed in the different metrics depending on the requirements.

Alternative test systems in the test strategy for the assessment of ENM

In Figure 2 alternative test systems are not integrated with exception of the screening tests in Step 1. The conventional endpoints used for risk assessment are selected with respect to the protection of populations and cover parameters such as reproduction, mortality, growth. Individuals are not considered. The alternative parameters reviewed in the literature usually address less complex reactions (e.g. determination of specific enzymes or gene activities) resulting often in an increased sensitivity. It is not always obvious whether an effect detected by a sensitive additional endpoint (e.g. indicators for oxygen stress) has an impact on the population level or indicates a compensation measure of the organism. Nevertheless, every additional parameter can provide additional information on ecotoxicity of ENM and can support the assessment. So far, it seems to be mainly a political decision whether the additional information is requested for the assessment of ENM. The advantage compared to the current procedure could be limited. However, research on the suitability of alternative endpoints is ongoing and final conclusions are not yet possible. In any case, in research, alternative endpoints play a major role by increasing the knowledge on the mode of action of ENM and improving their assessment.

However, there can be some specific effects which are not detected with the conventional endpoints but which might have an impact on the population level and as such might be of relevance for assessing the hazard of ENM. In the following such parameter are addressed.

Immunotoxicity / Genotoxicity

So far, the information on genotoxicity and immunotoxicity reviewed in the literature is obtained by in vitro-studies (e.g. test with blood cells or in fish cell culture) or by studies on microorganisms (e.g. Ames test) and therefore, the relevance for a population is not obvious. Effects which are detected in in-vitro-tests must not necessarily occur by an exposure of whole organisms. If it is verified that immunotoxicological and genotoxicological effects of ENM will affect stability and size of populations, these endpoints could be added to the test strategy. However, this is not considered to be an ENM specific problem. The reasons for modifying the basic test strategy for ENM (Figure 2) should also be reflected for conventional chemicals. Additionally, it has to be considered that information on genotoxicity and immunotoxicity has to be provided within the scope of the assessment on human toxicology and it has to be clarified whether additional information on ecotoxicological genotoxicity and immunotoxicity is necessary for the assessment.

Recommendation:

The knowledge on the significance of effects on immunotoxicity and genotoxicity caused by ENM in vitro and on the population level should be improved. Furthermore, the results have to be compared with the results obtained within the scope of studies on human toxicology. Based on this information it can be agreed on whether these parameter are a suitable addition to the ecotoxicological test strategy.

Bioaccumulation

Accumulation and even transgenerational transfer of ENM is detected. In the OECD expert meeting on ecotoxicology and environmental fate, among others the suitability of the OECD test guidelines dealing with accumulation (OECD TG 305, 315, 317) was discussed

(Kühnel & Nickel, 2014). For the TG on accumulation in fish the BCF (OECD TG 305) was considered to be not applicable for water phase and dietary spiking is favoured. So far, the knowledge on PC-parameters indicating accumulation of ENM is limited. Due to observed accumulation and transgenerational transfer with (i) concurrently insufficient information which ENM tend to accumulate and hence have to be tested and (ii) missing suitable test guidelines on accumulation, we have added the parameter “bioaccumulation” to the alternative test systems. Thus it becomes obvious that we consider this parameter as essential information and propose a simplified approach unless more comprehensive approaches covering uptake and elimination can be recommended.

Recommendation:

To get information on the accumulation potential of ENM we recommend a pragmatic procedure. We propose to determine the ENM concentration in the test organisms of the ecotoxicological tests at the end of the incubation period. This should be performed in the tests with aquatic and terrestrial oligochaetes. These organisms are already considered in accumulation studies and guidelines for the assessment of conventional chemicals are available. But also daphnids and fish embryos might be suitable candidates. In addition, we recommend the ENM-concentration determined also in ecotoxicological tests with plants (e.g. OECD TG 208 seedling and emergence, DIN EN ISO 22030 chronic test). This addition is justified by the large accumulation potential of ENM in sewage sludge and the agricultural use of sewage sludge in various countries and subsequently in an entry of ENM into agricultural soils. By this procedure, first information on uptake of ENM can be achieved. If more detailed results are required specific studies on bioaccumulation can be performed considering the discussions of the OECD expert meeting (Kühnel & Nickel, 2014) and – if already available – the TGs on accumulation of ENM. Furthermore, the entire results on accumulation obtained by the investigation of various ENM can be used to identify PC-parameter indicating bioaccumulation.

Multi-generation-tests

It cannot be excluded that effects become more pronounced if multi-generation-tests are performed. Additionally, recovery studies can provide interesting information (Völker et al., 2013). However, for regulatory testing the experimental effort should not be mandatory. Uncertainty is considered in the risk assessment, e.g. by assessment factors. Furthermore, we assume that the information obtained by multi-generation-tests and recovery studies is not nano-specific. It should also apply for conventional chemicals and for these chemicals the current risk assessment procedure has been successfully applied since many years.

Recommendation:

We do not regard the integration of multi-generation-tests in a test strategy for regulation as necessary (justification: see above).

Behavioural tests

In the reviewed literature, behavioural tests revealed to be quite sensitive. The test approach has the advantage that the results give information relating to the population level. However, so far, the information on the applicability on a wide range of ENM is limited. To extend the knowledge, the behavioural test with earthworms was studied in more detail in the experimental section of this project. A recommendation on such tests is provided in chapter 5.

Further test organisms

ENM agglomerate in aquatic systems, and increased concentrations in the sediment are expected (Gottschalk et al., 2009). The standardized test organisms *Chironomus riparius* and *Lumbriculus variegatus* develop in the sediment. It cannot be excluded that organisms living and grazing on the sediment are exposed to a higher extend compared to the standard test organisms. Many alternative test organisms have been applied for the assessment of ENM. In the reviewed literature mussels (*Mytilus galloprovincialis*) were used as test organisms to study ecotoxicity of TiO_2, carbon black, fullerene and SiO_2(Canesi et al., 2010b) [11].Other authors also published results on ecotoxicity and accumulation of various ENM on mussels (e.g.(Conway et al., 2014; Gagne et al., 2008; Gomes et al., 2011; Hull et al., 2011; Ringwood et al., 2010)).Additionally, snails such as *Physa acuta, Lymnaea stagnalis* or *Pyringa ulvae* might be of interest (e.g. (Bernot & Brandenburg, 2013; Justice et al., 2014; Khan et al., 2013; Musee et al., 2010; Oliver et al., 2014)).These organisms don't belong to the traditional test organisms. Organisms which are already used for the assessment of chemicals and which may be suitable are amphipods such as *Hyalella azteca* and *Gammarus pulex*. Also rooted submerged aquatic macrophytes (e.g. *Myriophyllum sp*.)(J. Glenn et al., 2012)may be of interest as they have a large surface and can filter ENM out of the water phase. Currently, information on the sensitivity of alternative organisms compared to the traditional ones is very limited.

Recommendation:

Knowledge on the suitability of the listed organisms for hazard assessment of ENM is missing. We recommend that the sensitivity of the listed organisms (sediment organisms, aquatic macrophytes) and of the standard test organisms (*C. riparius, L. variegatus*) are compared to decide on the suitability of further test organisms or the replacement of traditional organisms for the testing of ENM. By the selection of the test organisms the various living characteristics and feeding behaviours have to be considered.

5 Experimental section

5.1 Introduction

For conventional chemicals, it was demonstrated that the sensitivity of the avoidance behaviour with *Eisenia fetida* (ISO 17512-1) reaches or increases the sensitivity of reproduction tests (Hund-Rinke et al., 2005; Hund-Rinke & Wiechering, 2001) and in a laboratory comparison test the comparability of the results determined in various laboratories was proven (Hund-Rinke et al., 2003a). The test meets the criteria for a suitable screening test listed above (chapter 4.3) with (I) easy performance and (II) short test duration. However, the knowledge on the sensitivity of this test for ENM was still limited. To improve the information, the avoidance test was performed with five ENM from the OECD-Sponsorship Programme (CeO_2: NM-211, NM-212, ZnO: NM-111, CNT: NM-403, Ag: NM-300K) and with soluble $AgNO_3$. The results were compared with those of the reproduction test. The tests were performed in a natural sandy soil.

5.2 Materials and Methods

5.2.1 Nanomaterials

Various nanomaterials used in the OECD Sponsorship Programme were selected.

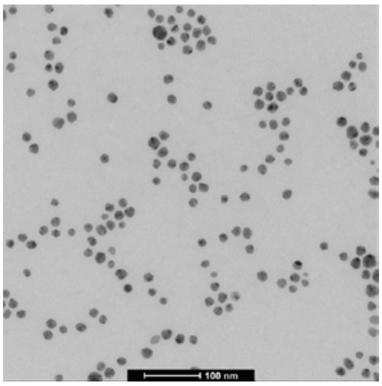
- $AgNO_3$: ---
- Ag: NM-300K
- ZnO: NM-111
- CeO_2: NM-211, NM-212
- CNT NM-403

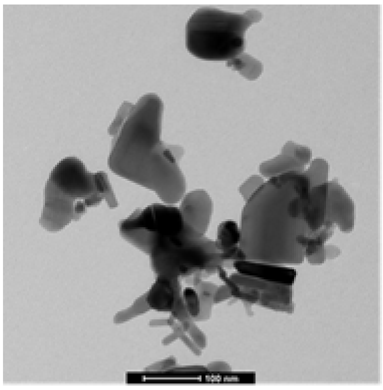
In Table 3 the basis for the selection and in Figure 3 - Figure 5 TEM-pictures are presented.

Table 3: Nanomaterials from the OECD Sponsorship Programme selected for the earthworm studies.

Material	ENM-Code	Justification
$AgNO_3$	---	Toxicity via ions; highest bioavailability; reference to Ag nanoparticles
Ag	NM-300K	Toxicity via ion release; locally increased concentration due to particular state
ZnO	NM-111	Toxicity via ion release; locally increased concentration due to particular state
CeO_2	NM-211, NM-212	poorly soluble nanomaterial (no or very low ion release)
CNT	NM-403	Carbon based nanomaterial

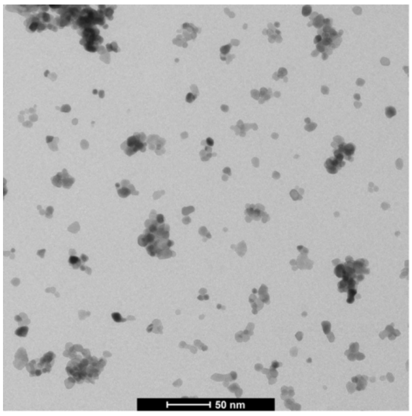
Figure 3: Representative micrographs of the selected Ag and ZnO nanomaterials, dispersed into double distilled water (TEM pictures by C O D A - C E R V A; Veterinary and Agrochemical Research Centre; Electron Microscopy; Groeselenberg 99; 1180 Bruxelles).

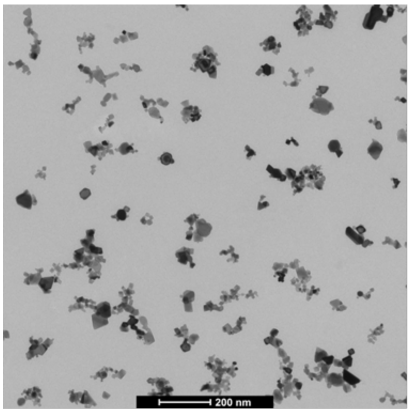




Ag – NM-300K: consisted of individual particles and agglomerates. In general, these particles were evenly distributed over the grid surface. The specimen consisted mainly of individual particles, and only a few agglomerates were detected. In addition, larger agglomerates were very rarely detected. Ag ENM were heterogeneously shaped, e.g. triangular-shaped. The primary particles of the nanomaterial had a size of approximately 20 nm, measured on the TEM images. The general morphology of the primary subunits of the ENM was nearly equiaxed and rounded, or slightly elongated. Their suggested 3D structure was spherical or slightly ellipsoidal. The agglomerates tended to be more or less equiaxed. One large nanoparticle (± 45 nm) was surrounded by one to eight smaller (18-20 nm) particles, possibly due to steric preferences or charge dependent interactions.	ZnO – NM-111 consisted mainly of aggregates and agglomerates. Primary particle sizes ranging from 10 nm up to 370 nm were measured. The general morphology of the primary particles of the ENM varied from circular or square to elongated and rectangular, suggesting a spherical, cubic, ellipsoidal, rod-like or even needle-like 3D structure. It is important to note, however, that some of the apparent differences in primary particle shape were the result of projection of similar particles with different orientations. The aggregates and agglomerates had a size ranging from 150 nm up to 2.5 µm. In most cases, the aggregates and agglomerates tended to have a dendritic structure.

Figure 4: Representative micrographs of the selected CeO_2-nanomaterials dispersed into double distilled water (TEM pictures by C O D A - C E R V A; Veterinary and Agrochemical Research Centre; Electron Microscopy; Groeselenberg 99; 1180 Bruxelles).





CeO_2 -NM-211 dispersed into double distilled water, consisted mainly of single particles and small aggregates. But large agglomerates occurred as well. A small variation in the size of the primary particles of the nanomaterial was found on all the TEM images. Primary particle sizes ranging from 4 nm up to 15 nm were measured. The primary particles had a similar shape. The general morphology of the primary particles of the ENM range from circular or slightly elongated to rectangular with rounded corners. This suggests a spherical, ellipsoidal or cuboid-like 3D structure. It is important to note, however, that the apparent differences in primary particle shape might have been the result of projection of similar particles with different orientations.	CeO_2 - NM-212 dispersed into double distilled water, consisted mainly of aggregates and agglomerates. Single particles were occasionally detected as well. Primary particle sizes ranging from 5 nm up to 75 nm were measured. The primary particles were heterogeneous in shape. The morphologies of the primary particles of the ENM were circular, triangular, rectangular or square. This suggests a spherical, pyramidal, cuboid, or cubic 3D structure. Some of the apparent differences in primary particle shape might have been the result of projection of similar particles with different orientations.

Figure 5: Representative micrographs of the selected CNT nanomaterials, dispersed into double distilled water containing 0.05% BSA (TEM pictures by C O D A - C E R V A; Veterinary and Agrochemical Research Centre; Electron Microscopy; Groeselenberg 99; 1180 Bruxelles).

CNT – NM-403 consisted mainly of agglomerates of CNTs, and single CNTs. The isolated tubes (primary particles) of the nanomaterial were not homogenous in size. The size distribution was very large as CNTs with a length ranging from 50 nm to 1.5 µm are detected on the images. The diameter of the CNT ranges from 5 nm to 25 nm. The general morphology of the isolated tubes (primary particles) of the ENM is rod-like, suggesting a tubular shape. However, the CNTs were not straight but were curved to varying degrees. Deformations, referred to as knots, in the tube shape were often observed. Figure 5 demonstrates the occurrence of non-CNT entities in the specimen. It is well known that metal catalysts are used in the production process of CNTs, and can remain in the samples as crystals. These catalysts (or other contaminants) can be embedded in the CNTs which results either in functionalization of the CNTs or deformation of the tubes. Consequently, non-CNT entities form part of the identity of the nanomaterial as processed and in use. The metal oxide contaminants will take up a large fraction of the weight of the sample, since they are more electron dense than the CNT.

5.2.2 Soil

The test and carrier soil for the application was RefeSol 01A, a loamy, medium-acidic, and lightly humic sand (pH 5.67; Corg 0.93%, sand 71%, silt 24%, clay 5%), which has been described in detail (Schlich et al., 2012). RefeSol 01A matches the properties as stated in various OECD terrestrial ecotoxicological guidelines (e.g., tests with plants and soil microflora). The soils were sampled in the field and were stored and treated according to good agricultural practice. They were stored outdoors in stainless-steel containers, red clover was sown on the stored soils, and no pesticides were used. Appropriate amounts of soil were sampled from the outdoor boxes one to four weeks before the test. If the soil was too wet for sieving, it was dried at room temperature to 20 to 30% of the maximum water-holding capacity (WHC_{max}), with periodic turning to avoid surface drying. If the tests did not start immediately after sieving, the soil was stored in the dark at 4°C under aerobic conditions (ISO Guideline 18512, 2007).

5.2.3 Application

The nanomaterials were applied by mixing the test material and air-dried carrier soil with the same physicochemical properties as the test soil. Enough ENM were added to the carrier to achieve the final test concentration when 5% carrier soil and 95% test soil were mixed to homogeneity. The soil was mixed with a spoon instead of a pestle to avoid modifying the ENM. Uncontaminated soil (at 20–30% WHC_{max}) was spread on a plate, and the spiked carrier soil was evenly distributed over the test soil before mixing. The mixed soil was adjusted to 55% WHCmax using deionized water. This procedure was performed for every test concentration. All concentrations refer to the dry matter of soil.

5.2.4 Earthworm reproduction test

We used *Eisenia fetida*, which has been cultured in our laboratory for more than 15 years. The earthworms were bred under defined conditions (1:1 mixture of cow manure and sphagnum peat at 20-28°C and a light:dark cycle of 16:8 h). Three days prior to the test, the earthworms were adapted to the test soil under experimental conditions (55% WHC_{max}), with free access to food. In the test, we only used earthworms with a clitellum and a wet mass of 300 to 500 mg.

All tests were carried out as described in OECD guideline 222, ''Earthworm reproduction test with *Eisenia fetida*'' (OECD TG 222, 2004), which allows the use of *E. fetida* and *E. andrei* as test organisms.

We filled polypropylene containers (Bellaplast GmbH) to a depth of approximately 5 cm with 625 g dry mass of soil (55% WHC_{max}) and then spread 40 g wet mass of cow dung (air dried, ground, and moistened before application) onto the surface. The cows were kept in an ethical husbandry. The tests were performed in eight replicates for the negative control and four replicates for each treatment. For chemical analysis with DGT devices, we prepared single analytical test replicates for each concentration to avoid exposing the earthworms to stress.

After overnight equilibration of the treated soils, 10 earthworms were added to each container and were incubated at 20-28°C with a light:dark cycle of 16:8 h (~700 lux). Once per week, the water content was checked gravimetrically, the evaporated water was replaced, and 20 g wet mass (corresponding to 5 g dry mass) of uncontaminated food was spread onto the soil surface in each container. After 28 d, the adult earthworms were removed and weighed, and after 56 d, the number of juveniles in each test container was counted.

Statistical calculations were performed with ToxRat Pro 2.10 software for ecotoxicity response analysis (ToxRat Solutions GmbH). For the median effective concentration (EC50) calculation, we use da probit analysis, assuming log-normal distribution of the values. For each concentration, we determined the percentage of mortality, the percentage of loss or gain in biomass of the adults, and the number of offspring produced in the test.

5.2.5 Earthworm avoidance test

The test was performed in a two-chamber system (Hund-Rinke et al., 2003b). As the test design is described in the ISO guideline (ISO Guideline 17512-1, 2008), only a short description of the test performance is given.

The containers were filled with soil up to a height of about 5–6 cm. Vessels with a surface of 200 cm^2 were used in analogy to the reproduction test. At the beginning of the test, the vessels were divided into two equal sections by a vertically introduced plexiglass divider. One half of the vessel was filled with test soil and the other half with control soil. Then the separator was removed, and ten adult worms of the species *Eisenia fetida* (weight: 300 – 600 mg) were placed on the separating line of the vessels. To prevent the worms from escaping, the vessels were covered with gauze permeable to light and air. For each concentration five vessels were applied. The vessels were incubated for 48 h at 20 ± 2°C. In analogy to the acute test and the reproduction test, the vessels were incubated at a day/night rhythm of 16/8 hours with a light intensity of 700 – 800 lux. The number of worms present in the contaminated soil and in the control was determined at the end of the incubation time. Worms stretching across the separating line were counted as 0.5, independent of the length of the body present in the two soils.

Differences between the control and the test soil were determined by statistical analysis. A soil was considered to be toxic when a statistically significant difference ($p < 0.05$) was determined. In addition, EC_{50}-values were calculated (ToxRat Professional, Version 2.10).

The tests with CeO_2 were additionally performed with aged soil. Spiked soil was incubated for 28 days at 20°C under aerobic conditions in the dark, followed by the test.

5.3 Results

5.3.1 Reproduction test

The results of the reproduction tests are presented in Table 4 - Table 10, the ECx-values are listed in Table 11. The validity criteria according to the TG were fulfilled in every test.

Validity criteria:

- each replicate (containing 10 adults) produced ≥30 juveniles by the end of the test
- the coefficient of variation of reproduction was ≤30 %;
- adult mortality over the initial 4 weeks of the test was ≤10 %.

NM-300K comprises of Ag and dispersant. We proved in a pre-test that the dispersant resulted in no significant difference to the control (Table 8). Therefore, the treated samples were tested against untreated soil.

The test substances differ in their ecotoxicity. The order of the substances with decreasing ecotoxicity is:

$AgNO_3$>NM-300K (Ag) >NM-211 (CeO_2) ≈NM-111 (ZnO)

NM-212 (CeO_2) and NM-403 (MWCNT) resulted in a statistical significant decreased reproduction activity but there was no concentration effect relationship. Comparable reproduction rates were achieved independent of the test concentration. For CeO_2 no literature data on effects on earthworm reproduction were available. Only a study with the *Caenorhabditis elegans* in aqueous test medium was found (Roh et al., 2010). Due to the different exposure this reference is not suitable to compare the results. Effects but missing effect relationships were observed in tests with double-walled carbon nanotubes-contaminated food and *Eisenia veneta*(Scott-Fordsmand et al., 2008). In the publication the missing effect-relationships are not addressed. Currently, we cannot explain this effect. But as it is observed also in further studies it may be specific for toxic insoluble or poorly water soluble (nano)materials.

ECx-values on reproduction could be determined for $AgNO_3$, NM-300K and NM-212 (Table 11). For NM-111 a slight inhibition was obvious (Table 4), however we could not determine an EC10-value due to statistical reasons. The number of offspring in the replicates of the different test concentrations overlapped. The difference in the mean values of the number of offspring in the four test concentrations was small and the replicates overlapped. NM-112 and NM-403 resulted in inhibitions of the reproduction, but no concentration-effect relationship was obtained, no EC-values could be calculated.

Besides the number of offspring increase of biomass of the adult worms was investigated. The weight change is calculated based on the total weight of the worms in every test vessel. The number of worms at test end is not considered. In every test the total weight of the worms in the treated samples showed a higher increase in biomass compared to the worms in the control soil. We observed a concentration of the worms in the food and an avoidance of the treated soil. Thus, a higher food uptake followed by a higher increase in weight cannot be excluded. The tests with NM-112 and NM-403 which run at the same time were an exception. Calculating the weight per surviving worm resulted only in a small increase in weight. The CeO_2-concentration of 50 mg/kg resulted even in strong decrease. The missing weight increase in this test cannot be explained as the applied food and test soil were comparable to the other tests.

Table 4: Results of the reproduction test with NM-111 (ZnO).

	Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Reproduction: number of offspring per vessel					
Mean value [1]	135	105 *	98 *	95 *	93 *
Standard deviation	9	11	12	14	13
Survival					
Number of animals per treatment [2]	73	36	38	40	34
Biomass					
Weight change (start – end) [%] [1, 3]	22	17	34	47	20

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control 80; treated 40. 3: number of dead animals at test end is not considered.

Table 5: Results of the reproduction test with NM-211 (CeO_2).

	Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Reproduction: number of offspring per vessel					
Mean value [1]	135	135	115 *	111 *	86 *
Standard deviation	9	21	13	18	19
Survival					
Number of animals [2]	73	40	40	40	36
Biomass					
Weight change (start – end) [%] [1, 3]	22	43	55	48	41

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control 80; treated 40. 3: number of dead animals at test end is not considered.

Table 6: Results of the reproduction test with NM-212 (CeO_2).

	Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Reproduction: number of offspring per vessel					
Mean value [1]	124	80 *	81 *	67 *	107 *
Standard deviation	9	12	13	28	7.9
Survival					
Number of animals [2]	75	37	37	24	40
Biomass					
Weight change (start – end) [%] [1, 3]	-6	0.6	3	-46	15

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control 80; treated 40. 3: number of dead animals at test end is not considered.

Table 7: Results of the reproduction test with NM-403 (MWCNT).

	Control	10 mg/kg	100 mg/kg
Reproduction: number of offspring per vessel			
Mean value [1]	124	100 *	99 *
Standard deviation	9	13	7
Survival			
Number of animals [2]	75	40	40
Biomass			
Weight change (start – end) [%] [1, 3]	-6	11	2

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control 80; treated 40. 3: number of dead animals at test end is not considered.

Table 8: Results of the reproduction test with NM-300K (Ag) – pre test.

	Control	Dispersant ENM-300KDIS (concentration comparable to amount in 200 mg/kg NM-300K)	60 mg/kg	120 mg/kg	200 mg/kg
Reproduction: number of offspring per vessel					
Mean value[1]	347	329	186 *	147 *	86 *
Standard deviation	27	4	22	24	18
Survival					
Number of animals [2]	40	40	40	40	40
Biomass					
Weight change (start – end) [%]	36	39	49 *	43 *	46 *

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control and treated 40.

Table 9: Results of the reproduction test with NM-300K (Ag) – main test.

	Control	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg	200 mg/kg
Reproduction: number of offspring per vessel						
Mean value[1]	341	252 *	209 *	220 *	158 *	97 *
Standard deviation	26	31	46	30	18	26
Survival						
Number of animals [2]	80	40	40	40	40	40
Biomass						
Weight change (start – end) [%][1]	40	62 *	62 *	61 *	67 *	62 *

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control 80; treated 40.

Table 10: Results of the reproduction test with $AgNO_3$.

	Control	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Reproduction: number of offspring per vessel					
Mean value[1]	347	261 *	202 *	158 *	107 *
Standard deviation	27	24	17	28	16
Survival					
Number of animals [2]	80	40	40	40	40
Biomass					
Weight change (start – end) [%][1]	36	58 *	55 *	59 *	56 *

1: Asterisk indicates statistical significance (0.05). 2: Number of introduced animals: control 80; treated 40.

Table 11: ECx values of reproduction test with ENM and $AgNO_3$.

	NM-111	NM-211	NM-212	NM-403	NM-300K	$AgNO_3$
EC50 [mg/kg] [1]	n.d. [2]	> 100	n.d.	n.d.	80.0 [33.6 - 413.3]	46.9 [40.7 – 53.6]
EC10 [mg/kg] [1]	n.d. [2]	24.4 [n.d.]	n.d.	n.d.	n.d.	10.9 [7.8 - 14.0]

1: n.d.: not determined due to mathematical reasons or inappropriate data; values in brackets: confidence interval. 2: no calculation of an EC10 due to an overlap in the number of offspring in the replicates of the various test concentrations; mean values indicate slight inhibition.

5.3.2 Avoidance test

The results of the avoidance tests are presented in Table 12 - Table 16, the ECx-values are listed in Table 17. The validity criterion according to the TG was fulfilled in every test.

Validity criterion:

- mortality or missing worms per test concentration ≤10 %

The ecotoxicity of the tested ENM differs. Depending on the ecotoxicity, either EC10 or an EC50 values were calculated. NM-403 (CNT) showed no toxicity at all.

The order of the substances with decreasing ecotoxicity is:

$AgNO_3$>NM-300K (Ag) >NM-111 (ZnO) >NM-212 (CeO_2) >NM-211 (CeO_2) >NM-403 (CNT)

Table 12: Results of the avoidance test with ZnO.

	Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
NM-111 (ZnO)					
Mean value [1]	5.4	6.0	4.8	4.7	3.7
Standard deviation	1.5	0.7	1.8	1.7	0.8

Table 13: Results of the avoidance test with CeO_2.

	Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
NM-211 (CeO_2) – immediately after spiking					
Mean value [1]	3.6	5.7	6.0	5.0	5.7
Standard deviation	1.6	1.9	2.3	2.0	1.9
NM-211 (CeO_2) – 28 d after spiking					
Mean value [1]	5.2	6.4	6.3	5.6	5.2
Standard deviation	1.8	1.5	1.1	1.7	2.3
NM-211 (CeO_2) – 28 d after spiking (2^{nd} experiment)					
Mean value [1]	4.6	---	4	---	5.4
Standard deviation	1.5	---	1.5	---	2.3
NM-212 (CeO_2) – immediately after spiking					
Mean value [1]	6.1	5.4	5.4	4.9	5.8
Standard deviation	1.0	1.2	1.1	2.1	1.6
NM-212 (CeO_2) – 28 d after spiking					
Mean value [1]	5.0	2.3	3.8	3.7	3.8
Standard deviation	0.7	1.6	1.8	1.9	0.4
NM-212 (CeO_2) – 28 d after spiking (2^{nd} experiment)					
Mean value [1]	4.6	---	5.2	---	3
Standard deviation	1.5	---	1.6	---	0.9

1: Number of worms in the treated compartment of the chamber; control: right test compartment.

Table 14: Results of the avoidance test with NM-403 (CNT).

NM-403 (CNT)	Control	10 mg/kg	100 mg/kg
Mean value [1]	3.6	6.2	4.7
Standard deviation	1.6	1.2	0.8

1: Number of worms in the treated compartment of the chamber; control: right test compartment.

Table 15: Results of the avoidance test with NM-300K (Ag).

NM-300K (Ag)	Control	1.2 mg/kg	3.7 mg/kg	11 mg/kg	33 mg/kg
Mean value [1]	5.9	3.7	2	0.8	0.7
Standard deviation	1.2	1.6	1.3	0.7	0.6

1: Number of worms in the treated compartment of the chamber; control: right test compartment.

Table 16: Results of the avoidance test with $AgNO_3$.

$AgNO_3$	Control	6.25 mg/kg	12.5 mg/kg	25 mg/kg	50 mg/kg
Mean value [1]	5.2	0.5	0.5	0.1	0
Standard deviation	1.0	0.7	0.6	0.2	0

1: Number of worms in the treated compartment of the chamber; control: right test compartment.

Table 17: ECx values of avoidance test with ENM and $AgNO_3$.

	NM-111	NM-211	NM-212	NM-403	NM-300K	$AgNO_3$
EC50 [1]	>100	>>100	n.d. [2]	>100	1.9 [0.9 – 3.1]	<< 6.25 (mean valued of 0.5 worms in treated soil in lowest test concentration)
EC10 [1]	33.5 [n.d.]	>100	n.d. [2]	>100.	n.d.	n.d.

1: n.d.: not determined due to mathematical reasons or inappropriate data; values in brackets: confidence interval. 2: no calculation of an EC10 due to missing concentration-effect-relationship; but reduced number of offspring in all treatments.

We performed an additional experiment with CeO_2 using aged soil. Soil was spiked with CeO_2 (NM-211, NM-212), incubated over 28 days and used for an avoidance test. To validate the results, this test was performed twice. In both tests, NM-212 caused avoidance behaviour of the worms after the aging period. About five to six worms were detected in the soil treated with NM-212 in the test started immediately after spiking. After the aging period, about two to four worms were determined in the treated soil in the first test. There was no concentration dependency in the avoidance behaviour, but the percent standard deviation decreased with the highest standard deviation in the soil with the lowest test concentration (68 % referring to the mean value of worms in the treated soil) and the lowest standard deviation at the highest test concentration (12 % referring to the mean value of worms in the treated soil). We conclude that the decreasing standard deviation indicates the increasing homogeneity of spiking due to the larger amounts of ENM in the higher test concentrations. In the second test a difference in the effects depending on the test concentration was detected with a higher effect at the higher test concentration. We explain the observation as follows:

1. Effect only after aging:
   This result indicates a modification of NM-212 during the aging period. The underlying reason may be a changed crystalline structure (Wohlleben, personal communication).
2. Missing concentration-effect-relationship in the first test:
   The missing concentration-effect-relationship indicates a comparable concentration of toxic compounds responsible for the avoidance in all treatments. We assume that mainly water soluble transformation products are detected by the sensory structures of the earthworms which are located in the integument. If the ENM themselves would be detected, we would expect a concentration-effect-relationship. It is well known that soluble metals interact with soil components such as humic materials or clay. From the results, we assume that the transformation products of the ENM interact with soil components resulting in a comparable bioavailable concentration independent of the test concentration. The underlying processes are still unknown and have to be clarified.

No avoidance behaviour was observed with NM-211. The missing ecotoxicity of NM-211 was independent of the aging period. About four to six worms were observed in the treated soil in both tests.

NM-211 is smaller and has a larger surface area than NM-212. Based on the size, we would expect the ecotoxicological potential of NM-211 increases the ecotoxicity of NM-212. The missing effect may be due to the modification of the crystalline structure of NM-212 during aging which is less pronounced for NM-211 (Wohlleben, pers. communication).

5.3.3 Comparison: reproduction test - avoidance test

We compiled the EC-values of both test types to compare their sensitivity for ENM (Table 18). In Table 19 the results are presented in a simplified way.

Table 18: ECx values of reproduction test and avoidance test with ENM and $AgNO_3$.

	Endpoint	Reproduction test	Avoidance test
$AgNO_3$	EC50 [mg/kg] [1]	46.9 [40.7 – 53.6]	<<6.25
NM-300K (Ag)	EC50 [mg/kg]	80.0 [33.6 - 413.3]	1.9 [0.9 – 3.1]
NM-111 (ZnO)	EC10 [mg/kg]	n.d. [2]	33.5 [n.d.]
NM-211 (CeO_2)	EC10 [mg/kg]	24.4 [n.d.]	>100 (pristine and aged ENM)
NM-212 (CeO_2)	EC10 [mg/kg]	n.d. [3]	Pristine ENM: >100 Aged ENM: n.d. [3]
NM-403 (MWCNT)	EC10 [mg/kg]	n.d. [3]	n.d.

1: n.d.: not determined due to mathematical reasons or inappropriate data; values in brackets: confidence interval. 2: no calculation of an EC10 due to an overlap in the number of offspring in the replicates of the various test concentrations; mean values indicate slight inhibition. 3: no calculation of an EC10 due to missing concentration-effect-relationship; but reduced number of offspring in all treatments (first test) and only two test concentrations (second test).

Table 19: Comparison of reproduction test and avoidance test with ENM and $AgNO_3$.

	Reproduction test [1]	Avoidance test [2]
$AgNO_3$	✓	✓✓
NM-300K (Ag)	✓	✓✓
NM-111 (ZnO)	✓	✓
NM-211 (CeO_2)	✓	Pristine, aged: ---
NM-212 (CeO_2)	✓[2]	Pristine: --- aged: ✓[3]
NM-403 (MWCNT)	✓[2]	---

1: ✓ effect; ✓✓ increased effect compared to ✓ in the reproduction test; --- no effect. 2: missing concentration-effect-relationship. [3]: missing concentration-effect-relationship in one of the two tests.

For ion releasing ENM the avoidance test seems to be a suitable screening test. The sensitivity of the avoidance behaviour for NM-300K exceeds the sensitivity of the reproduction test of about a factor of 10. The results support the findings of Shoults-Wilson et al. (2011a, b). Additionally, we observed for NM-111 avoidance behaviour comparable to the sensitivity of the reproduction test.

The two CeO_2 ENM and the MWCNT showed a differing behaviour. The materials are insoluble or poorly soluble, but at least the CeO_2 NM-212 can transform during a longer incubation period (Wohlleben, pers. communication). This transformation may be the reason for the observed avoidance behaviour after an aging period of the NM-212. We observed an effect neither in the reproduction test nor in the avoidance test applying MWCNT. Missing effects in both tests were also observed for C60-ENM (Li & Alvarez, 2011). However, a concentration-dependent effect on reproduction of *Lumbricus rubellus* was observed in a study performed by van der Ploeg et al. (2011). The maximum test concentration was 154 mg/kg. In contrast to the studies mentioned above they applied an ENM containing soil extract for spiking the soils. It cannot be excluded that the different spiking procedures (van der Ploeg: wet spiking; Li and Alvarez: dry spiking) affected the bioavailability and toxicity of the ENM.

McShane et al. (2012) tested TiO_2 which is also an insoluble or poorly water soluble ENM on earthworm reproduction and avoidance. They observed an effect in the avoidance test but not in the reproduction test at a test concentration of 10 g/kg. This test concentration exceeded the test concentrations used in our study (maximum 100 mg/kg) and may be responsible for the difference in the sensitivity observed in the studies with insoluble or poorly water soluble ENM. We consider a test concentration of 10 g/kg as unsuitable for ecotoxicological tests. In the OECD test guidelines which were developed for the hazard assessment of soluble chemicals a maximum test concentration of 1000 mg/kg is recommended. Therefore, the observed differences in the sensitivity of the avoidance and reproduction test between our study and the study on TiO_2 should not be overestimated.

The worms are fed in the reproduction test and thus increase in weight. The weight change of the adult worms in the reproduction tests with ENM was more pronounced in the treated soil compared to the control soil as indicated by an avoidance of the treated soil. This behaviour was also observed for CeO_2 indicating a sensing of toxic compounds which was not obvious in the avoidance test performed immediately after spiking. Therefore, the avoidance test after an aging period was performed. After the aging period, NM-212 caused an ecotoxicological effect. NM-211 did not affect the worms, independent of the aging period, although an effect was observed in the reproduction test. This reveals that the basis of the CeO_2 ecotoxicity in the reproduction and avoidance test is currently not understood.

5.3.4 Conclusion: suitability of the avoidance test in the test strategy

The avoidance test with its short incubation period provides interesting information and has to be used target-oriented. Depending on the ENM-properties different information can be achieved.

- The test cannot be recommended as general screening component of the testing strategy on ecotoxicity of ENM in regulation so far. False negative assessments cannot be excluded.
- The test provides information on the ecotoxicity of ion releasing substances and can be used for the ranking of such substances. The test can underestimate ecotoxicity if it is performed immediately after spiking with ENM which are quite poorly soluble or which become toxic by aging.
- The test can be used to give indications on the fate of ENM in the complex matrix soil which cannot be described by chemical methods so far as respective analytical procedures are still missing.
    - Information on formation of toxic transformation products during aging: Aging of the soil, performance of the avoidance test several times during the aging period and increasing avoidance behaviour followed by constant avoidance behaviour provide respective information. Constant avoidance behaviour over time indicates stable conditions concerning the bioavailability of the ENM.
      Justification based on the experimental section of this project: modifications of ENM can be slowly (toxicity of NM-212 resulted in ecotoxicological effects after an aging period of 4 weeks) and due to the short test period of 2 days the test provides information on the current state of the ENM.
    - Information on potential interaction of ENM and soil components: Ecotoxicity but missing concentration-effect relationships indicate that the bioavailable portion of the contaminant is independent of the test concentrations. Interactions with soil components or formation of large agglomerates are assumed.
      Justification based on the experimental section of this project: comparable effects of NM-212 in the avoidance test although the test concentrations ranged from 1.25 mg/kg to 100 mg/kg.
    - The test may be used to give first indications on the fate of ENM in the complex matrix soil. Currently chemical methods are missing to describe the ENM in a complex environmental medium such as soil or sediment. However, care must be taken in the interpretation of the results. Exemplarily, it has to be considered that reduced ecotoxicity can be the consequence of decreased bioavailability due to sorption.
      Justification based on the experimental section of this project: NM-212 resulted in ecotoxicological effects after an aging period; the ecotoxicity of NM-211 did not change over time.

Further research is needed to understand the interactions and to correctly interpret the results. This includes the investigation of

- further ENM differing in the type and size
- further ENM differing in stability
- coated and non-coated ENM
- aged and pristine ENM

- We recommend further research to clarify the observed missing concentration-effect relationship for insoluble or poorly water soluble ENM. For these ENM, the test concentrations resulted in comparable effects. Therefore, following aspects should be considered:
    - Testing of lower concentrations to get information on the range between "no effect" and "maximum effect".
    - Further work on the spiking procedure.
      We had compared wet and dry spiking for Ag and TiO_2(Hund-Rinke et al., 2012). Based on the results we preferred dry spiking due to concentration-effect-relationships which we did not obtain with wet spiking. The results obtained with C-60 show different effects (Li & Alvarez (2011); van der Ploeg et al. (2011)). It should be clarified whether the suitability of dry and wet spiking depends on the ENM.

6 Fate test strategy

6.1 General considerations

The current status of (inter)national discussions and scientific publication on in-depth insight into environmental fate processes of ENM as well as potentially needed adaptation of existing or development of new experimental tests describing environmental behaviour of ENM is by far less comprehensive compared to the ecotoxicological section. However, prior to suggesting a fate test strategy the available literature was reviewed in order to obtain a complete picture on the state-of-the-art (literature review performed in 2013; some additions in 2013 and 2014). To avoid the exclusion of important citations or aspects, no focus was lead on any special group of ENM such as silver and TiO_2, on any test design used in fate assessment, or on any fate-related endpoint.

The publications were evaluated with respect to their suitability for the fate test strategy:
Is the published test method, test design, model or test strategy suitable for the fate test strategy to be developed in the project?
Are certain, selected aspects valuable for use in the test strategy?
Should additional methods not considered so far be taken into account?

6.2 Literature review concerning fate

For the identification of relevant publications following sources were used:

- Data base of research literature - Science direct
- Web of knowledge

The following keywords were used:

- „Nanoparticles“, „Nanomaterial“ + „solubility“
- „Nanoparticles“, „Nanomaterial“ + „dissolution“
- „Nanoparticles“, „Nanomaterial“ + „release“
- „Nanoparticles“, „Nanomaterial“ + „partitioning“
- „Nanoparticles“, „Nanomaterial“ + „adsorption“, „sorption“, „desorption“
- „Nanoparticles“, „Nanomaterial“ + „sedimentation“
- „Nanoparticles“, „Nanomaterial“ + „transport“
- „Nanoparticles“, „Nanomaterial“ + „mobility“
- „Nanoparticles“, „Nanomaterial“ + „distribution“
- „Nanoparticles“, „Nanomaterial“ + „stability“
- „Nanoparticles“, „Nanomaterial“ + „hydrolysis“
- „Nanoparticles“, „Nanomaterial“ + „degradation“
- „Nanoparticles“, „Nanomaterial“ + „transformation“
- „Nanoparticles“, „Nanomaterial“ + „bioaccumulation“
- „Nanoparticles“, „Nanomaterial“ + „bioavailability“
- „Nanoparticles“, „Nanomaterial“ + „fate“
- „Nanoparticles“, „Nanomaterial“ + „PEC-assessment“
- „Nanoparticles“, „Nanomaterial“ + „PEC-models“; „PEC-modelling“
- „Nanoparticles“, „Nanomaterial“ + „risk assessment“

For a more systematic evaluation of the retrieved citations an allocation of key words to few environmental fate parameters was performed as follows:

Table 20: Key words used in literature search and allocation to topics for “fate”.

Description of parameter in compiled literature	Allocated to
solubility	solubility
dissolution	
release	
octanol / water partition coefficient	partitioning
stability in aquatic systems	Stability in the (aquatic) environment, transformation, degradation
hydrolysis	
dispersion stability	
Degradation	
transformation	
adsorption	Mobility and transport in porous media; sorption / desorption to soil, sediment, and sludge
sorption	
desorption	
sedimentation	
transport potential in porous media	
mobility in soil	
distribution (in sediments)	
deposition	
Bioaccumulation	Bioaccumulation
Fate model	PEC-assessment, PEC-models
Material flow modelling	
Probabilistic approach	
Risk assessment	Risk assessment approaches
Life cycle assessment	

Results of literature search are presented on the following pages. References are sorted according to the environmental parameters presented in the right column of Table 20.

Table 21: Compilation of the reviewed literature on fate of nanomaterials.

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
Topic: solubility					
1.	Review: nano-Zn. nano-Ag given as examples	Review article: Indirect testing by ecotoxicity testing, solubility used to explain toxicity	AAS	Aschberger et al. (2011)	Definition of endpoint described; citations mainly deal with effects testing, solubility used to explain tox-tests results. Relevant in particular for inorganic ENM
2.	Fullerene C60	Commonly used test systems for logPow and solubility testing (OECD-TG)	HPLC UV/VIS (336 nm)	Jafvert et al. (2008)	OECD 105, 107, 117; slightly adopted.
3.	Al_2O_3 nanoparticles	10 L outdoor microcosms, lake water, test duration 210 days. Specific "endpoint": Dynamics of particle behaviour in natural aquatic systems in terms of mean hydrodynamic diameter, specific surface area, <u>dissolution</u> of soluble aluminium.	Size and shape be TEM; particle size analysis by dynamic light scattering; ion dissolution kinetics of Al^{3+} by ICP-OES	Pakrashi et al. (2012)	Behaviour can be described by application of respective methods with sufficient precision. Results showed a rapid particle aggregation, a decrease in specific surface area and a significant increase of soluble Al. From these results the needed conclusions can be drawn with sufficient precision
4.	Gum arabicum (GA) and polyvinylpyrrolidone (PVP) coated nano silver	Aquatic microcosms, different types of: surface water, water and sediment, water and aquatic plants. Specific "endpoint": <u>Dissolution</u>, fate, stability in aquatic systems.	Particle size characterization: TEM; electrophoretic mobility: phase analysis light scattering (PALS); oxidation state: X-ray absorption near edge spectroscopy; total Ag analysis: ICP-MS; particle size distribution: AF4 multidetection analysis	Unrine et al. (2012)	Dissolution and aggregation behaviour of nano-silver was investigated using a broad set of analytical methods. Excellent combination of methods for determination of the ENM status in aquatic systems

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
5.	Ag ENM	Organic-coated silver nanoparticles (Ag ENM having particle diameters ranging from 5 to 80 nm, synthesized using various methods, and with different organic polymer coatings including poly(vinylpyrrolidone) and gum arabicum) analysis were used to determine the local structure around Ag and evaluate changes in crystal lattice parameters and structure as a function of ENM size.	Size, morphology: transmission electron microscopy (TEM), X-ray absorption fine structure (XAFS) spectroscopy, synchrotron-based total X-ray scattering, and pair distribution function (PDF)	Ma et al. (2012)	Dissolution of Ag measured by using a broad variety of methods. Results are consistent with the finding that Ag ENM solubility can be estimated based on TEM-derived particle size using the modified Kelvin equation for particles in the size range of 5–40 nm in diameter.
Topic: partitioning					
6.	Carbon nanotubes (CNT)	Ecotoxicological testing	CNT-characterization by TEM, scanning electron microscopy (SEM), Zetasizer Nano ZS	Petersen et al. (2010)	Substantial differences in apparent distribution coefficients of two CNTs were observed. However, results on bioaccumulation studies (earthworm) suggest that traditional distribution behaviour-based Kow approaches are likely not appropriate for predicting CNT-bioaccumulation
7.	Fullerene C60	Artificial sediment prepared as described in OECD 225. Spiked with C60 dissolved in toluene, thoroughly mixing, air-dried for 48 h, extracted by normal shaking procedure	HPLC-UV/VIS (285 nm)	Wang J. et al. (2011)	Easily and commonly used method for test system setup and analysis

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
Topic: stability in the (aquatic) environment, transformation, degradation					
8.	- (Review)	Main parameter is agglomeration / aggregation which is influenced by a combination of many factors (natural organic matter (NOM), colloidal clay. ionic strength (IS), pH and inherent ENM properties such as surface charge). Example for consequences for environmental behaviour: in natural water low pH and high NOM result in ENM stabilisation and thus long-range transport potential is high. Fate and transport of ENM in porous media (soil and sediment) is affected by agglomeration size, charge and agglomeration rate in the transport media	- (Review)	Aschberger et al. (2011)	Definition of endpoint and consequences for environmental behaviour described. Relevant for organic and inorganic ENM
9.	Metal oxide nanoparticles	Powder aggregates were dispersed in water. Neither ultrasound nor chemical dispersants could break them up into primary nanoparticles. It is difficult to disperse dry commercial nanoparticles. Except for silica, other nanoparticles rapidly aggregated in tap water due to electric double layer compression	ENM characterization in water by: dynamic light scattering (for average particle sizes), microflow imaging (for particles sizes and corresponding number concentrations).	Zhang et al. (2008)	Not to be used for drawing conclusions with respect to test methods. Too specific topic
10.	TiO_2 (Evonik P25 and Hombikat UV-100)	The aquatic systems were obtained by addition of Na^+, Ca^{2+}, Cl^-, SO_4^{2-}, Suwannee River Natural Organic Matter (NOM), and sodium diphosphate. NOM-addition did not interfere turbidity measurements	Particle size and electrophoretic mobility by Zetasizer Nano ZS; concentration of nano-TiO2 by turbidity measurements	von der Kammer et al. (2010)	Presented methodology can serve as a basis for development of standardized methods for comparing the behaviour of different nanoparticles in aquatic systems.
11.	ZnO Nanomaterials	bulk environmental samples used	Isotopic analysis (MC-ICP-MS)	Larner and Rehkämper (2012)	Applicable for inorganic ENM; highly sophisticated analytical methods; tracing purposes; bulk environmental samples

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
12.	Nano zero-valent zinc	Lab studies, aqueous solutions, humic acid added; stability in water tested	TEM (morphological analysis); UV-scanning (sedimentation); fluorescence spectrometry measurement and fluorescence quenching analysis (interactions between ENM and humic acid)	Dong and Lo (2012a)	Highly sophisticated methods, special topic of surface description and description of interaction between ENM and humic material, probably no use for routine measurements of inorganic ENM (ICP-MS or AAS might be better).
13.	Fullerene C60, water stable	Aqueous system; ENM suspension and dissolved ozone	Analysis of form of products: ^{13}C-NMR, MS, FTIR, UV_VIS and XPS	Fortner et al. (2007)	Aqueous reactivity of fullerene-based materials must be considered appropriately for accurate assessment of their transport. To be used for fate and potential environmental risk assessment.
14.	Ag ENM	Influence of pH, ionic strength, and humic substances on the stability of carbonate-coated Ag-ENM systematically investigated in 10 mM carbonate and 10 mM MOPS buffer, and in filtered natural freshwater.	Nanoparticle tracking analysis, dynamic light scattering, and ultraviolet–visible spectroscopy.	Piccapietra et al. (2012)	Agglomeration measured under various conditions. Combination of conditions gives comprehensive information on behaviour to be expected also in the natural environment.
15.	TiO_2 ENM	Structural stability in media mimicking the environment or the conditions of use was tested. Aging by aggregation, desorption of Si from the coating and effects of water at pH=5 (low ion strength) on $Al(OH)_3$ layer were tested.	Spectroscopic methods	Auffan et al. (2010)	Tests give reliable information on stability under environmental conditions

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
16.	TiO_2 ENM	Contributions of natural organic matter (NOM) and bacteria to the aggregation and deposition of TiO_2 nanoparticles in aquatic environments were tested. Transport experiments with ENM were conducted in a microscopic parallel plate system and a macroscopic packed-bed column using fluorescently tagged E. coli as a model organism and Suwannee River Humic Acid as a representative NOM.	Labelled with fluorescein isothiocyanate allowing particles and cells to be simultaneously visualized with a fluorescent microscope.	Chowdhury et al. (2012)	Systems to test aggregation and deposition are near to natural conditions, influence of environmental parameter tested.
17.	TiO_2 ENM	Determination of diffusion coefficients of TiO_2 nanoparticles having a nominal size of 5 nm. The effects of a various concentrations of the Suwannee River Fulvic Acid (SRFA) and the roles of pH and ionic strength were evaluated.	Fluorescence correlation spectroscopy	Domingos et al. (2009)	
18.	TiO_2-ENM	Colloidal stability tests on commercially relevant titanium dioxide nanoparticles (Evonik P25) in well-controlled synthetic waters covering a wide range of pH values and water chemistries, and also in standard synthetic (EPA) waters and natural waters. Results from matrix testing in well-controlled batch systems allow predictions to be made on the behaviour in the broader natural environment.		Ottofuelling et al. (2011)	Provides the basis for a testing scheme and data treatment technique to extrapolate and eventually predict nanoparticle behaviour in a wide variety of natural waters
19.	TiO_2-ENM	ENM behaviour and toxicity towards algae in the presence of secondary treated wastewater effluent was tested. TiO_2-ENM fate and toxicity in secondary treated effluent was compared to that in reference organic matter, specifically Suwannee River humic acid.	laser diffraction, ICP-MS	Neale et al. (2014)	
20.	Various ENM	review	review	Aschberger et al. (2011)	Definition of degradation both for organic and inorganic ENMs

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
21.	Silver nanoparticles	review	review	Levard et al. (2012)	Partly applicable to test strategy since review without detailed description of methods
22.	Silver, titanium dioxide and C60 (fullerene) nanomaterials	Sequencing batch reactors were used simulating a several weeks WWTP	ICP-OES (metals); UV/VIS (fullerenes and fullerols)	Wang et al. (2012)	Highly sophisticated test system. simulation experiments
23.	Silver nanoparticles	Freshwater mesocosms (3.66 m x 1.22 m x 0.8 m) simulating an emergent wetland environment were dosed. Dosing was by spraying either to the water column or to the terrestrial soil. Terrestrial soil was planted. Duration of the experiments: 18 months	Graphite furnace AA (for Ag in soils. sediments. plants). ICP-MS (for Ag in fish and insects)	Lowry et al. (2012)	Highly sophisticated higher tier approach
24.	Silica nanoparticles	The fate of silica nanoparticles (SiO_2NPs) during simulated primary wastewater treatment, by measuring, in real time, the colloidal behaviour of SiO_2NPs in wastewater (sewage) was measured. The effects of surface functionality on SiO_2NP fate in wastewater was investigated by comparing both non-functionalized (uncoated or "bare") SiO_2NPs and SiO_2NPs functionalized with a thin coating of a non-ionic surfactant.	Small-angle neutron scattering	Jarvie et al. (2009)	Well balance comparison of different products
25.	Ag-ENM	The behaviour of metallic silver nanoparticles (Ag-ENM) in a pilot wastewater treatment plant (WWTP) fed with municipal wastewater was tested. The treatment plant consisted of a non-aerated and an aerated tank and a secondary clarifier. Ag-ENM were spiked into the non-aerated tank and samples were collected from the aerated tank and from the effluent.	ICP-MS, TEM, X-ray	Kaegi et al. (2011)	Applicable for risk assessment

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
Topic: mobility and transport in porous media; sorption / desorption to soil sediment, and sludge					
26.	TiO_2 nanoparticles	Columns (id: 2.5 cm. length 20 cm) filled with 12 different soils	Digestion of ENM followed by ICP-OES	Fang et al. (2009)	Set-up comparable to (aged) soil column leaching, can be used for sorption and transport consideration
27.	CeO_2-nanoparticles	Laboratory-scale activated sludge system	Inductively coupled plasma-optical emission spectroscopy (OES) for Ce-analysis. Stability of CeO_2 nanoparticles in dispersions by particle size distribution and zeta potential	Gómez-Rivera et al. (2012)	Sophisticated sorption studies and activated sludge studies can be performed easily for metal based nanoparticles. Good analytical methods available.
28.	60 nm polystyrene microspheres. fullerene C60, surfactant wrapped nanotubes (SWNT)	Screening technique, deep well plate columns (96-position 2-mL deep well plate) packed with different porous media (fresh water sediment. high purity quartz), 2 mm hole in the bottom, 20 µm frit, column length 3.4 cm	Suspended SWNT and C60 mass by UV-VIS; polystyrene ENM concentration by fluorescence emission intensity	Bouchard et al. (2012)	Well suited for rapidly screening the mobility of engineered ENM
29.	C60 fullerene	Various sorbents (charcoal. soil. dissolved organic matter) was used; C60 fullerene added, extracted from aqueous phase by toluene.	Concentration measurements by UV-spectrometry (336 nm)	Li et al. (2008)	Sorption testing of organic ENM has to be discussed carefully, since results depend on clean-up method.
30.	nanoscale zero-valent iron (nZVI); stabilized by polyelectrolytes	Laboratory experiments including column and batch sedimentation studies. Influence of polyelectrolytes on mobility was tested	TEM for surface; Atom absorption for total iron concentration; evaporation method for solids content	Jiemvarangkul et al. (2011)	Useful to elaborate the possibilities and limitations of methods to describe the mobility in the environmental compartment soil.
31.	Aqu/C60 and water soluble C60 pyrrolidine tris acid C60-PTA	Packed columns, column leaching	Concentrations determined by UV/VIS (322 nm for C60 and 300 nm for C60-PTA)	Zhang W.et al. (2012)	Applicable test system
32.	ZnO nanoparticles	Packed column experiments	Determination of zeta-potential and particle sizes	Jiang et al. (2012)	Can be used for determination of transport potential; comparable to other soil-leaching experiments (OECD-TG)

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
33.	Nano zero-valent iron (NZVI)	Nano zero-valent iron (NZVI) was synthesized and loaded by Cr and As. respectively. Excess metal ions were washed-off. Desorption experiments by overhead shaking for 24 h (comparable to other desorption experiments).Cr and As were measured in the solution.	AAS-analysis of metals	Yin et al. (2012)	Results might be depending on test design.
34.	Fullerenes, silver, gold and polystyrene nanomaterials	Sorption of ENM to freeze-dried, heated activated sludge as required by OPPTS results in low sorption compared to fresh sludge. This might be attributed to surfactant like materials (proteins, phospholipids…) liberated from the sludge when freeze-dried and heated, which alter the sorption behaviour of ENM	ICP-OES (silver, gold), HPLC (fullerene), hydrodynamic diameter by PALS. zeta potential by dynamic light scattering particle sizer	Kiser et al. (2012)	Sorption of ENM to activated sludge has to be determined by using fresh activated sludge; thus, OPPTS as it stands has to be modified. Fresh activated sludge reasonably predicts full-scale performance for titanium removal. As long as differently treated sludge surfaces are compared the method is appropriate. In case ENM inherent properties are to be determined the method is debatable as the results depend on clean-up methodology.
35.	8 and 15 nm silica nanotubes	Column transport experiments	TEM, Zetasizer Nano ZS	Wang et al. (2012)	Higher particle number concentration led to lower relative retention and greater surface coverage. Smaller ENM resulted in higher relative retention and lower surface coverage. Study highlights the importance of ENM concentration and size on their behaviour in porous media. Improved equation for surface coverage calculation using column breakthrough data.

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
36.	Fullerene nano-C60 particles (nC60)	Batch system to measure Kd-values; different water/ethanol mixtures were used to vary the polarity of the solvent system	HPLC of fullerene in soil extracts. Zeta potential and particle size in suspension determined	Forouzangohar et al. (2011)	Importance of hydrophilic forces in controlling the sorption behaviour of fullerene particles which are stabilized in water dominated solvent mixtures. The general validity of partitioning mechanisms and Koc-modelling approaches in describing and estimating the sorption of nC60 particles in soil is questioned. It may be valid for more un-polar dominated solvent mixtures (> 60% ethanol).
37.	CeO_2 ENM	CeO_2 ENM in water saturated porous media. column packed with sand	?	Li et al. (2011)	Model can be applied to assess the risk of CeO_2-nanoparticles transport in contaminated ground water
38.	Fullerene C60 nanomaterial	small membrane based soil cell	Spectrophotometer, UV-measurements	Chen L. et al. (2012)	Limited applicable for testing; column leaching method preferred as they refer to OECD TG
39.	Nanosized sulphate-functionalized polystyrene latex particles. nanosized carboxyl-modified latex particles. carboxyl-modified CaSe/ZnS quantum dots	Column (8 cm x 1.6 cm) experiments using biofilm coated or extracellular polymeric substances coated sand	ENM-characterization: dynamic light scattering (for hydrodynamic diameter). laser Doppler velocimetry in conjugation with phase analysis light scattering (for electrophoretic mobility)	Tripathi et al. (2012)	Good methodology to test the influence of particle surface chemistry on affinity to coated sand. i.e. to environmental particles
40.	Carboxymethyl cellulose stabilized zero-valent iron nanoparticles	Column experiments	Iron concentrations by AAS	Feng et al. (2009)	Applicable, gives good results, can be adapted to. e.g., OECD TG on (aged) leaching

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
41.	Fullerene nanoparticles (nC60)	Column transport experiments; size: 0.6 x 10 cm; filled with sand and sandy soil, leaching by aqueous solutions of different ionic strength and switching from NaCl to $CaCl_2$. Flow velocity also was modified during experiment.	?	Zhang L. et al. (2012)	Can be used to investigate the influence of ionic strength and of flow velocity on leaching depending on the porous medium
42.	Untreated metal oxide nanoparticles (Fe_3O_4, CuO, ZnO, TiO_2)	Column experiments; spherical glass beads used as porous material	TEM, ICP-AES, UV_VIS	Ben-Moshe et al. (2010)	Of limited use since glass beads were used
43.	Amphiphilic colloids (e.g., monodispersed hydrophobic chloromethyl latex). different diameter	Column breakthrough experiments. size: 4.6 cm id, 10 cm length	?	Zhuang et al. (2005)	Not relevant for test strategy as the columns were filled with amphiphilic colloids and breakthrough (e.g. of bromide) was tested
44.	Carboxyl functionalized single-walled carbon nanotubes (SWNT)	Columns filled with quartz sand; id: 1.6 cm, height: 6.3 cm. SWNT applied, ionic strength of mobile phase modified and deposition and breakthrough, respectively, determined	Breakthrough curves established based on chromatographic measurements of SWNTs	Jaisi et al. (2008)	Can be used to test basic processes; for fate-testing soil as porous medium is more appropriate
45.	Aluminium Nanoparticles	Column leaching; columns filled with sand and soil, respectively.	Zeta potential and hydrodynamic diameter (by laser Doppler electrophoresis and by dynamic light scattering). concentration by UV_VIS (700 nm); leachates were measured	Darlington et al. (2009)	Good methodology, applicable

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
46.	P25 titanium dioxide (nano-TiO_2) as sorbent	Interaction between the heavy metal and natural organic carbon coated nanomaterial. Adsorption of Cd(II) on humic acid (HA) coated TiO_2 was investigated. Adsorption isotherms of Cd(II) to TiO_2 and HA-TiO_2 were described by Freundlich model	C, N and H-content analysed by elemental analyser. Nano-TiO_2 and HA-TiO_2 surfaces characterized by X-ray photoelectron spectroscopy; HA, nano-TiO_2 and HA-TiO_2 characterized by FT-IR; Cd(II) in solutions analysed by atom absorption spectrometry (AAS)	Chen Q. et al. (2012)	Used Analytical methods are state-of-the-art with different specificity levels. As nanomaterials are used as sorbent only and sorption properties of Cd(II) in a special environment are tested the citation is of minor to no relevance for the test strategy.
47.	Nano TiO_2	Field-scale investigations	Analytical scanning electron microscopy; analytical (scanning) transmission electron microscopy (TEM); ICP-AES for Ti in methanolic extracts of soil and sewage sludge	Kim et al. (2012)	Appropriate for exact quantification
48.	Ag ENM	Mobility and deposition of capped Ag-ENM on silica surfaces were characterized over a wide range of pH and ionic strength conditions including seawater and freshwater. Citrate and PVP were used as trapping agents. Capped Ag ENM and silica surfaces were negatively charged under these environmentally relevant conditions resulting in net repulsive electrostatics under most conditions. The steric repulsion introduced by the capping agents significantly reduced aggregation and deposition. The presence of NOM further decreased deposition on silica. Ag ENM found to be mobile under these conditions	ICP-AES	Thio et al. (2012)	Specific method but seems applicable for mobility measurements

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
49.	Iron nano-particles	Study used a well-defined porous medium for transport analysis of iron particles. A porous pattern-regularly-etched glass model allowed for quantification and visualisation of mobilization and deposition of iron nano-particles during transport. Mobility and deposition of ENM under various environmental conditions. e.g., addition of organic matter, was quantified; transport is described	Particle concentration determined by UV spectrophotometer (508 nm)	Wang Q. et al. (2011)	Highly specific method; seems not to be widely applicable
50.	Nano zero-valent iron	Fate of As(V)-treated NZVI (As-NZVI by examination of desorption potential of As. Desorption induced by phosphate (= extraction by phosphate). Desorption kinetics were determined and calculated.	GF-AAS	Dong et al. (2012b)	Same concerns against adsorption / desorption as mostly discussed
51.	TiO_2 ENM	Evaluation of transport of two different TiO_2 nanoparticles (P25 TiO_2 and synthesized TiO_2). Laboratory flume, column, and batch experiments were conducted to investigate the processes dominating the transport of TiO_2 nanoparticles between streams and streambeds and to characterize the properties of these nanoparticles under different physicochemical conditions. A process-based colloid exchange model was applied to interpret the observed transport behaviour of the TiO_2 nanoparticles.		Boncagni et al. (2009)	Same concerns against adsorption / desorption as mostly discussed, however, due to combination of methods interpretable results are achieved.
52.	CeO_2 ENM	Using ultrafiltration (UF), dissolution, and non-equilibrium retention (Kr) values of citrate-coated (8 nm diameter) CeO_2 ENM and partitioning (Kd) values of dissolved CeIII and CeIV were obtained in suspensions of 16 soils with a diversity of physicochemical properties.	ICP-AES	Cornelis et al. (2011)	As non-equilibrium retention was determined the fact that processes of ENM in the environment are non-equilibrium processes is addressed.

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
53.	Ag ENM	Investigation of the mobility of four silver nanoparticles (Ag ENM) stabilized using different capping agents and representing the common stabilization mechanisms as well as surface charging scenarios in reactive and nonreactive porous media. The results highlight the importance of both the stabilization mechanism and capping agent chemistry as key factors governing the transport of Ag ENM in the environment.	ICP-AES	El Badawy et al. (2013)	Comprehensive study aiming at mimicking different scenarios.
54.	Ag ENM	To understand the relative impact of humic (HA) and fulvic fraction of NOM on the stability and mobility of silver nanoparticles (Ag ENM), a combination of dynamic light scattering and quartz crystal microgravimetry with dissipation monitoring was used.	combination of dynamic light scattering and quartz crystal microgravimetry with dissipation monitoring	Furmanet al. (2013)	Very much related to the combination of various techniques, might be useful to assess the relative influence of a parameter (in this case humic acid)
55.	Ferrihydrite ENM	The study focuses in particular on the influence of flow rate and ionic strength on particle mobility. Column tests were performed under constant or transient ionic strength. A simple relationship is proposed for the estimation of travel distance with changing flow rate and ionic strength.		Tosco et al. (2012)	
Topic: bioaccumulation					
56.	Isotopically modified Copper ENM	Bioaccumulation in snails	Determination of zeta-potential; TEM; hydrodynamic size; atom force microscopy (AFM); X-ray diffraction; ICP-AES	Misra et al. (2012)	Comprehensive efforts taken for accumulation studies and various dependencies. However, applicable and reliable results

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
57.	review	review	review	Aschberger et al. (2011)	Methods exist to test bioaccumulation. Applicable to organic and inorganic ENM. Examples: C60 and CNT observed to adhere to the external surfaces of organisms; the same is valid for nano Ag. Uptake of nano-Zn by roots. no translocation
58.	Carbon nanotubes (CNT)	Ecotox testing	CNT-characterization by TEM, scanning electron microscopy (SEM), Zetasizer Nano ZS	Petersen et al. (2010)	Substantial differences in apparent distribution coefficients of two CNTs were observed. However, results on bioaccumulation studies (earthworm) suggest that traditional distribution behaviour-based Kow approaches are likely not appropriate for predicting CNT-bioaccumulation.
59.	Nanoscale metal oxides (TiO_2, CeO_2, ZnO)	Semistatic exposure of Zebrafish and Rainbow trout	Water and tissue samples analysed by TEM and environmental scanning electron microscopy (ESEM) with energy-dispersive X-ray analysis (EDX). ENM exposure levels in tank and tissues by ICP-MS and ICP-OES	Johnston et al. (2010)	Adequate methods to determine fate and uptake of unmodified nanoscale metal oxides
60.	Arsenic bearing phases	monitoring	-	Grosbois et al. (2011)	Not applicable since monitoring approach
61.	C60 suspended in aqueous systems	Not further specified as review	Transformation of C60, transformation products measured by NMR, IR, MS and XPS methods	Isaacson et al. (2009)	Still lack of analytical methods; this has the potential to generate misleading results (publication of 2009!).

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
Topic: PEC assessment, PEC models					
62.	TiO_2, ZnO, Ag, CNT, Fullerenes	PEC based on a probabilistic material flow analysis from a life-cycle perspective. PEC is calculated by probabilistic density functions. PECs are expressed as "simulated modes" (=most frequent values).	Not applicable	Gottschalk et al. (2009)	Deals with uncertainties and thus reflects the fact of lacking information
63.	nano-TiO_2	PEC modelled by stochastic stationary substance / material flow modelling. Applicable to any substance with a distinct lack of data.	Not applicable	Gottschalk et al. (2010)	Deals with uncertainties and thus reflects the fact of lacking information
64.	Engineered nanoparticles (ENM)	Review on information on ENM release along the life-cycle of a product. Particularly sewage sludge, wastewater, and waste incineration are major flows through which ENMs are released into the environment. Reliable data are missing on release during production, application amount and empirical information on release coefficients for all life-cycle stages and environmental compartments.	Not applicable	Gottschalk and Nowack (2011)	
65.	Engineered nanoparticles	Design and evaluation of an ENM fate model capable of incorporating environmental complexity to predict realistic environmental concentrations of ENM. Model is based on a designated fate model for ENM in surface waters first developed by (Praetorius et al., 2012). As in the original model, heteroaggregation between the ENPs and suspended particulate matter (SPM) is assumed to be a key process determining ENM transport. Realistic conditions were introduced by defining variable SPM composition and concentration along the river and time dependent emissions.	Not applicable	Sani-Kast et al. (2014)	Model is based on the fact that fate processes of ENM are non-equilibrium processes. Thus, the model is a kinetic model. Valuable basis for all further model developments.

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
66.	Engineered nanoparticles	Due to the fundamentally different properties of ENMs compared to organic pollutants, the concepts and models valid for organic pollutants cannot simply be used for ENMs. A new concept for environmental fate models for ENMs is presented. The environmental fate of ENMs is dominated by aggregation, transformation and sedimentation processes. In contrast to organic chemicals whose environmental fate is largely governed by equilibrium partitioning, the fate of ENMs is exclusively kinetically controlled, because ENMs do not reach thermodynamic equilibrium but are present in the environment as unstable suspensions.	Not applicable	Praetorius (2014)	Model is based on the fact that fate processes of ENM are non-equilibrium processes. Thus, the model is a kinetic model. Excellent basis for all further model developments.
Topic: test strategies					
67.	nanopesticides	Concept to consider environmental fate of nanopesticides. Differentiation between: very slow / low release of active ingredient (decoupled) ->colloid transport, NC properties to be considered intermediate release (kinetic conditions) very rapid release (equilibrium conditions) -> solute transport, a.i. properties to be considered	Not applicable	Kah and Hofmann (2014a)	May serve as a basis for the development of a more generalised test strategy valid not only for nanopesticides.
68.	nanopesticides	Adaptations of current exposure assessment approaches (experiments and models) are needed to account for ENM specifics. The article provides a framework to identify research priorities.	Not applicable	Kah et al. (2013)	Conclusions are drawn which are applicable to other ENM and thus useful for any test strategy

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
69.	nanoformulation of atrazine	Regulatory protocols defined in the OECD TG were applied to determine the fate properties of a nanopesticide in two agricultural soils. It was concluded that more detailed investigations on bioavailability and durability of nanopesticides are necessary and will require the development of novel methods suitable to address both, the “nano” and “organic” characteristics of polymer-based nanopesticides.		Kah et al. (2014b)	Very valuable to be used in the context of RA and experimental test strategy
70.	Nanopesticides	Main focus is on assessing whether the presence of nanoformulation introduces potential differences relative to conventional active ingredients. The proposed changes in the test methodology, research priorities, and recommendations would facilitate the development of regulatory approaches and a regulatory framework for nanopesticides.		Kookana et al. (2014)	Valuable basis for the test strategy; can be generalised to ENM other than nano-pesticides.
Topic: Risk assessment (RA) approaches					
71.	TiO_2-ENM	To appreciate the uncertainties regarding the nano TiO_2 potential impacts and risks on the environment Life Cycle Assessment (LCA) and Risk Assessment (RA) approaches were combined. Since high uncertainties remain concerning the fate and the effect of nanoparticles, a probabilistic approach such as Bayesian network is used. Structure of the network: nodes (variables) and arrows (relationships between variables), probability tables of each variable.		Adam (2014)	Good basis for RA concepts dealing with uncertainties.

No.	Test material	Test system	Analytical method	Author / year	Remarks, conclusions
72.	ENM	Risk associated with exposure to engineered nanomaterials will be determined in part by the processes that control their environmental fate and transformation. It was concluded that risk associated with the use of ENM cannot be determined for pristine ENM only but has to consider their alterations and transformations in the environment.		Nowack et al. (2012)	Profound comparison of advantages and disadvantages in the use of pristine versus altered ENM for environmental RA.

6.2.1 Interpretation of results

The following overview summarizes and evaluates the most important results of the literature search. The literature is listed according to a set of topics which are relevant for the development of test strategies for ENM. The evaluation was done under the aspect of the suitability of the described test methods, models, or test strategies for their use in the fate test strategy which was developed in this project.

Solubility

Five references were found related to this topic. More literature is available covering the solubility of engineered nanomaterial (ENM) in one or the other way. However, the selection gives an impression on the current status of research with respect to the complexity of the test systems and the implementation of this parameter in test strategies.

OECD-test guidelines which are developed for chemicals (OECD 105) have been slightly modified to be used with fullerenes (Jafvert et al., 2008 [2]). Such modified test guidelines might give valuable results in case ENM of comparable nature (e.g. fullerenes, CNTs) are tested with identical methodological approaches. In this case the results might be used for a comparison and ranking of structurally comparable ENM but are not suitable to elucidate the ENM intrinsic properties[2].

Specific aspects of solubility such as the combination of analytical methods are addressed in the literature (Ma et al., 2012 [5]; Unrine et al., 2012 [4]). An “indirect testing” of solubility by use of ecotoxicological tests was described by Aschberger et al. (2011) [8]. Such an approach seems to be applicable in case the aspect of bioavailability is also considered.

<u>Result of evaluation of test methods with respect to their suitability for the test strategy and recommendations:</u>

The most promising test approaches are those which consist of complex systems mimicking the environmental conditions (Pakrashi et al., 2012 [3]; Unrine et al., 2012 [4]). Such approaches consider the complex nature of ENM and their complex interactions with the environment.

Partitioning

Two references specifically dealt with the partitioning of ENM between octanol and water (Petersen et al., 2010 [6]) as well as sediment and water (Wang J. et al., 2011 [7]). It is obvious that results from tests on the partitioning between water and octanol phase are not suitable to investigate the ecotoxicity of ENM - in this case CNT – (Petersen et al., 2010 [6]). Wang J. et al. (2011) [7] described a more complex system to determine the partitioning between sediment and water. Artificial sediment was prepared as described in OECD 225. It was spiked with C60 which was previously dissolved in toluene. The spiked sediment was thoroughly mixed, air-dried and thereafter extracted by a shaking procedure. The authors discussed this method as being easily applicable and commonly used.

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[2] „Intrinsic property“: property of an ENM which is independent from any other factors such as the amount tested, other test parameter or environmental parameter. The expression is used herein in the sense of an „absolute“ property rather than a „comparative“ property.

Result of evaluation of test methods with respect to their suitability for the test strategy and recommendation:

It is commonly accepted that guidelines which are based on partitioning processes are not suitable for ENM since non-equilibrium processes between ENM and the surrounding media occur.

Stability in the (aquatic) environment

Under the topic "stability in the aquatic environment", agglomeration / aggregation as well as colloid stability are covered.

The approaches described might be too specific (e.g. Zhang et al., 2008) [9] to be used as test strategy. Zhang et al. (2008) describe the behaviour of powder aggregates which were dispersed in water, treated by sonification or chemical dispersants to break them up into smaller particles. It was concluded that it is difficult to disperse dry commercial nanoparticles.

Several articles (Dong et al., 2012b [50]; Piccapietra et al., 2012 [14]; Chowdhury et al.; 2012 [16]; Ottofuelling et al., 2011 [18]) deal with the influence of the aquatic environmental conditions such as pH-value, ionic strength, humic acid content on the stability of ENM in the aquatic environment. Further research is required to investigate the interactions between the aquatic environment and ENM.

Experimental approaches which are based on modified standard test guidelines (von der Kammer et al., 2010 [10]) yield valuable results for comparing the behaviour of various nanoparticles in aquatic systems. However, they do not result in the description of intrinsic properties. Modifications of standard tests e.g. by the addition of natural organic matter sampled from a natural river or creek, should be considered.

Even more useful to describe the stability of ENM in natural aquatic systems is the use of media mimicking the respective environmental conditions (Auffan et al., 2010 [15]). This can in particular be achieved by using a complex laboratory test system such as model waste water treatment plants (Wang et al., 2012 [22]; Jarvie et al., 2009 [24]) or fresh water mesocosms (Lowry et al., 2012 [23]).

Result of evaluation of test methods with respect to their suitability for the test strategy and recommendation:

ENM are complex systems (more than commonly used chemicals) which interact with the complex system environment. Complex test systems are thus required to further elucidate the environmental behaviour of ENM.

Though the conclusion on the need of complex systems is commonly accepted in the scientific community none of the cited references explicitly deals with this issue in detail.

Mobility and transport in porous media; sorption / desorption to soil sediment, and sludge

Most of the articles listed in table 21 under this topic deal with the mobility and transport of ENM in porous media and soil, rather than in the aqueous compartment, although sedimentation is an important process in the water.

ENM stability during experiments and their characterization and quantification is usually addressed. It is well known that the size and size distribution are of major importance for the environmental behaviour of ENM and should thus be characterized and well documented in each experiment. It is recommended that besides size or size range a further characterization of ENM shape or nature and surface properties should be made (Kah et al., 2013 [68]).

It is well known that the concept of sorption which is based on distribution coefficients, and which is of major importance for the description of solutes transport in soil, is not appropriate for ENM. However, ENM association with soil is a non-equilibrium process, as it is also in other environmental compartments. Processes, which in addition have to be considered, are dispersion, aggregation, deposition and re-mobilization (Kah et al., 2013 [68]). Kah et al. (2013 [68]) summarized that it is evident, that most experimental set-ups might affect the form in which ENM occur. In that particular article nanopesticides were mentioned, however, the conclusions are true for all ENM. The authors mentioned the example of the soil to solution ratio which is used in batch experiments according to OECD 106: this is by far lower compared to the real environment and thus provokes aggregation or dissolution of the nano-pesticide which does not occur in the environment. Though Kah et al. (2013 [68]) focused on nano-pesticides their statements are applicable to other ENM as well.

Only few authors have tried to adopt and modify OECD 106 which has mostly failed due to the limited interpretability of the results (Li et al., 2008 [29]; Yin et al., 2012 [33]; Kiser et al, 2012 [34]; Forouzangohar et al., 2011 [36]; Chen L.et al., 2012 [38]; Dong et al., 2012a [12]; Boncagni et al., 2009 [51]). Forouzangohar et al. (2011) [36], for example, investigated the importance of hydrophilic forces in controlling the sorption behaviour of fullerene particles which were stabilized in water dominated solvent mixtures. The authors questioned the general validity of partitioning mechanisms and Koc-modelling approaches in describing and estimating the sorption of nC60 particles in soil. It was stated that it may be valid for more nonpolar dominated solvent mixtures, e. g, containing more than 60 % ethanol.

Kiser et al. (2012) [34] investigated the sorption of ENM to activated sludge as required by OPPTS. However, sorption of ENM has to be determined by the use of fresh sludge, and thus, the OPPTS methods as it stands needs to be modified. As long as differently treated sludge surfaces are compared, the method is appropriate. However, in case the ENM inherent property is to be determined, the method is questionable as the results depend on the clean-up methodology. Furthermore, solution ratio (see above) has to be taken into account as stated by Kah et al. (2013 [68]).

Other authors (Bouchard et al., 2012 [28]; Chen L. et al., 2012 [38]) suggested methods to screen the mobility potential of ENM. They used, for example, deep well plate columns or membranes to determine the ENM distribution and mobility. Such approaches, indeed, might be useful for a screening or ranking of ENM of similar size and surface properties. However, they are not appropriate for the determination of inherent properties.

Processes of ENM in the environment are non-equilibrium processes. This was addressed by Cornelis et al., 2011 [52] who determined the non-equilibrium retention (Kr) values by the use of ultrafiltration and dissolution.

<u>Result of evaluation of test methods with respect to their suitability for the test strategy and recommendation:</u>

Most appropriate is the use of experiments mimicking the environmental situation. In particular these are soil column experiments, e.g. as described in OECD 312. Accordingly, most of the publications deal with soil column experiments. Columns are of different size and packed with various porous media such as sand, quartz beads, charcoal, and soils of a broad range of properties. Most importantly, various techniques, in particular analytical techniques, should be combined in order to obtain a comprehensive and reliable picture of the ENM mobility in porous media and soil. Guidance is needed to consider specific information needs on ENM in the OECD 312.

Bioaccumulation in aquatic organisms

Few references are available describing the applicability of bioaccumulation tests for ENM. A review article (e.g. Aschberger et al., 2011) [57] stated, that methods exist to test bioaccumulation of organic and inorganic ENM. In contrast, Isaacson et al. (2009) [61], mentioned that analytical methods are still lacking which may lead to misleading results. However, the latter citation is of 2009, and analytical methods have been further improved since.

Another author (Misra et al., 2012) [56] took comprehensive efforts to experimentally determine the bioaccumulation of nano-Cu in snails. The semistatic exposure of zebra fish and rainbow trout to nano-TiO_2, nano-CeO_2 and nano-ZnO yielded reliable information on fate and uptake of unmodified nanoscale metal oxides (Johnston et al., 2010) [59].

<u>Result of evaluation of test methods with respect to their suitability for the test strategy and recommendation:</u>

Generally, the determination of bioaccumulation needs to account for the fact that uptake- and distribution-processes are kinetically driven.

PEC-assessment, PEC-models

Basically two aspects and approaches are dealt with in PEC assessment:

- Much information on input parameter is lacking and thus models are based on uncertainties.
- Fate processes of ENM are non-equilibrium processes and thus models are kinetic models.

Gottschalk et al. (2009, 2010) [62, 63] calculated PECs by the use of probabilistic density functions. PEC-values were based on probabilistic material flow analyses from a life-cycle perspective and were expressed as simulated modes, i.e. the most frequent values. Gottschalk et al. (2011) [64] gave a review on information on ENM release along the life-cycle of a product. Sewage sludge, waste water and waste incineration were identified as major paths of entry into the environment. Again, it was stated that reliable data are missing on the quantity of emissions into the environment during production and usage. This situation can be overcome to some extent by the already mentioned probabilistic density functions.

Environmental fate processes of ENM which are mostly aggregation, transformation and sedimentation processes are non-equilibrium but kinetic processes, because ENMs do not reach thermodynamic equilibrium but are present in the environment as unstable suspensions (Praetorius et al., 2014 [66]; Sani-Kast et al., 2014 [65]).Thus, conventional distribution models based on equilibrium processes such as the fugacity models developed by Mackay are not applicable. The authors designed and evaluated an ENM fate model which is capable to incorporate the environmental complexity to predict realistic environmental concentrations of ENM.

Result of evaluation of test methods with respect to their suitability for the test strategy and recommendation:

Equilibrium processes can be excluded almost completely with regard to the fate processes of ENM. Thus, the use of kinetic models is essential in PEC-assessment.

Test strategies

M. Kah and her co-workers (e.g., Kah et al., 2014a [69]) described a valuable concept which differentiates between various scenarios for the transport of nanopesticides in soil. Such a concept seems to be applicable to other ENM, at least to colloidal ENM. The concept differentiates between release situations of the active ingredient from the colloidal nanopesticide:

- very slow / slow release of active ingredient (decoupled)
  -> colloid transport, nanopesticides properties to be considered
- intermediate release (kinetic conditions)
- very rapid release (equilibrium conditions)
  -> solute transport, a.i. properties to be considered

However, it has to be defined, what exactly is meant by “very slow /slow”, “intermediate” and “very rapid” release.

This concept was further developed and described in detail by Kookana et al. (2014) [70] for nanopesticides and the soil compartment as path of entry into the environment:

Starting with the generally assumed durability of the nanopesticide in soil a differentiation between short lived and long lived nanopesticides in the soil compartment is made. In case of short lived products the nanopesticide is handled as a conventional active ingredient (a.i.). In case of long lived nanopesticides the fate and effects in soil have to be determined for the nano-form. The latter needs respective analytical methods. In case of long lived nanopesticides fate and effects not only in the initial compartment (in this case the soil compartment) but also in receiving compartments (surface water, groundwater) is to be considered. From fate studies in soil it is known whether nanopesticides are mobile / immobile and persistent / non-persistent. Based on this knowledge it has to be decided with which component (conventional a.i., nano-a.i. complex or free ENM acting as the a.i.) aquatic fate and effects studies should be carried out. Again, studies on nano-a.i. complexes and free ENM require specific analytical methods.

The authors also present a tiered approach for the analysis and characterization of nanopesticides. The tiers comprise:

Tier 1: a.i. exposure determination.

Tier 2: Minimum ENM-a.i. characterization (ultrafiltration or high speed centrifugation of sample).

Tier 3: Detailed ENM-a.i. characterization (low speed centrifugation of sample and ultrafiltration or high speed centrifugation of supernatant).

Tier 4: Complete ENM-a.i. characterization (tier 3 plus dispersion of filter retentate or pellet into aqueous solution and nano-characterisation methods).

In Kah et al. (2014b [69]) the fate of nanoformulated atrazine was investigated and results compared to those for conventional atrazine. It was concluded that more detailed investigations are needed on bioavailability and durability of nanopesticides and will require the development of novel methods suitable to address both, the “nano” and “organic” nature of the pesticides.

Result of the evaluation of the publication with respect to its suitability for the test strategy:

The concept of a tiered approach is very valuable to be further considered in the development of the test strategy. Tiered approaches generally focus on relevant aspects and thus are straight forward. In particular, the consideration of the stability of ENM properties in the environment is worth to be integrated in the test strategy. By doing so, those ENM which, once released into the environment, rapidly lose their properties can be identified and evaluated as conventional chemicals, which is cost and time effective.

Risk Assessment (RA) approaches

Only a few references deal with the risk assessment of ENM. These comprise:

- Comparison of RA of conventional substances and RA of ENM (see under paragraph “test strategies”: Kookana et al., 2014 [70]).
- Dealing with uncertainties and limited input information.
- Integration of ENM alteration and transformation in the RA.

Uncertainties regarding the potential impacts and risks associated with ENM were discussed by Adam et al., 2014 [71]. The authors combined life-cycle assessment (LCA) and RA approaches. Because high uncertainties remain concerning the fate and effects of ENM probabilistic approaches are needed. A Bayesian network was used.

Nowack et al. (2012) [72] concluded that risk due to ENM cannot be determined exclusively for pristine ENM but has to consider alterations and transformation in the environment.

Result of the evaluation of the publication with respect to its suitability for the test strategy:

The existing publications are a valuable basis for further consideration in the development of a tiered risk assessment approach. In particular dealing with limited input information is a method of choice.

6.3 Status of OECD-discussion on fate

The OECD working party on manufactured nanomaterials (OECD WPMN) hold an expert meeting from 29th – 31st January 2013 at the German Federal Press Office in Berlin. Among others, the OECD Expert Meeting on Ecotoxicity and Environmental Fate discussed on the applicability of existing test methods, which have been developed for conventional chemical substances, on manufactured nanomaterials. With respect to test methods on fate the OECD Expert Meeting on Ecotoxicity and Environmental Fate drew the following conclusions (Kuehnel and Nickel, 2014):

Fate and degradation in water

- A decision tree beginning with dispersion and dissolution (pH, NOM etc.) has to be developed. A tiered approach is needed as it also is necessary as first steps before sediment / soil testing.
- The current OECD Dissolution TG is NOT applicable.

- Biodegradation TGs which base on oxidation of organic carbon (e.g., BOD-determination) are applicable in few cases only. Other methods are needed which can detect and measure the biodegradation of the organic coating.

Bioaccumulation (water)

- The BCF approach of TG 305 not applicable since no partitioning takes place.
- Dietary exposure as given in OECD 305 needs to be used since equilibrium is not reached and thus a BCF cannot be determined. Also, the calculation of biomagnification factors requires other models for calculation.
- Problems with analytical detection in particular in tissues
- The determination of the uptake rate is challenging, since ENM might be only attached to the surface of the test organism but not incorporated. Thus, the internalization rate is hard to determine.
- In-vitro tests as pre-screening?
- Research necessary on uptake of ENM by filtering organisms (mussels, crustaceans)

Adsorption / desorption in soil and sediment

- Both processes, adsorption and desorption are directed by porosity, charge and NOM, but NOT by the organic carbon content as it often is the case for conventional chemical substances.
- It is expected that – due to size and charge – the mobility of nanomaterials in soil is limited.
- It is needed to determine the size distribution of applied and eluted ENM.
- The application of dispersion is more preferable than dry spiking.
- OECD 106 is not applicable and should be replaced by another test for ENM.
- OECD 312 is generally applicable (preamble/ guidance necessary).

Bioaccumulation (Soil/Sediment)

- OECD 315 and 317 generally applicable
- The bioaccumulation factor (BAF) is an appropriate endpoint, however, specific guidance needed
- Wet spiking mostly preferable (may be reasons why not)
- Test purged and unpurged worms

6.4 Environmental fate: overall conclusions based on OECD WPMN and literature search

From the literature screening and the conclusions drawn by the OECD WPMN (January 2013) most important conclusions are:

- Published results have to be evaluated carefully with respect to the reliability and robustness of the analytical methods applied. Publications without exact description of the applied analytical methods should not be taken into account for use in the integrative test strategy.

- In particular for the determination of size and size distribution a set of different analytical methods should be applied to obtain the most comprehensive information. But generally, the use of more than one analytical method seems to be useful to account for the complex nature of ENM.
- Most experimental set-ups are likely to affect the form in which ENM occur and might yield a result that is not representative of the behaviour under realistic environmental conditions.
- Tiered testing and risk assessment strategies are appropriate to account for the complex nature of ENM. They also account for the fact that some ENM might be short lived in the environment. In that case, risk assessment should be based on the “free”, conventional substance (e.g. on the free metal ion or the dissolved active ingredient in case of nanopesticides) – if that occurs.
- Risk assessment should be based on information on aged rather than on pristine ENM since that accounts for the durability of the ENM in the environment.
- The more complex the considered environment matrix is, the more environmental parameters interact with ENM which themselves are of complex nature. Consequently, the experimental test design needs to reflect this: The more complex the tested compartment is, the more should the experimental set-up mimic the real environment.
  E.g., on a first tier, fate and degradation in water can be investigated relatively easily by the use of a “standard water” and the determination of dissolution (new TG needed), dispersion stability (new TG needed), and the measurement of the stability of the organic coating (new or modified TG needed). On higher tiers, mesocosm studies might be valuable tools.
  In contrast, fate and degradation in soil, even on the first tier, should be investigated using a soil leaching study (OECD 312) mimicking the vertical transport through the soil. By comparing the results of fresh and aged soil leaching studies, even the aging can be taken into account.
- The development of new test methods or the interpretation of existing methods should be related to the protection target.
  It should – for example – be discussed as to whether a modified OECD 106 (sorption to soil) is even needed or whether it can completely be skipped and replaced by OECD 312 (soil column leaching). In this case, the aim of protection is groundwater protection. That means, that the portion of ENM which is eluted from the column is relevant for groundwater exposure and thus, sorption constants are not at all to be used for the groundwater risk assessment.
- It is worth to combine risk assessment and life-cycle assessment approaches.
- PEC assessment has to face the fact that fate processes (mainly aggregation, transformation and sedimentation processes) are kinetically dominated and non-equilibrium processes. Respective models (such as mentioned by Praetorius, 2014) have to be used.
- One approach to account for lacking information on input parameters for PEC models is to deal with uncertainties.

6.5 Test strategy for fate assessment

The test strategy as is stands for the fate assessment is shown on the following pages. Life-cycle aspects and the effects assessment part are not presented. The complete test strategy is shown in the introduction, whereas details for the effect assessment are presented in chapter 4.3.

Tier 0 (ENM to be tested):

On tier 0 of the test strategy the durability of the ENM in the initial compartment into which it has been released is screened. For that screening any information on the loss of ENM properties is used. Such information should be available (at least to some extend), e.g., from the manufacturer collecting it in the course of product design and development.

Figure 6: Test strategy for the fate of ENM.

Tier 0

Screening for durability of ENM in initial compartment

Information on loss of ENM properties

Medium - high durability

Information on:
parameters determining high to medium durability, size of initial compartment, amount released, production volume

Tier 1

fate	effects
• Testing of pristine ENM on: dispersion stability, kinetics on dissolution, modification of coating, further physico-chemical properties • Use of models and assessment procedures (*default models due to legal background*) • Result: n-PEC $_{ini}$ • Result: transport to secondary compartment possible / not possible	

↓ ↓

Predicted environment risk quotient in initial compartment: n-PEC $_{ini}$ / n-PNEC> 1;transport to secondary compartment possible

↓

Tier 2: refinement

Tier 2

fate	effects
• Testing of pristine <u>and</u> aged ENM on: transformation, transport / mobility (*modified or new OECD TG*) • n-PEC $_{ini\ refined}$ (*based on experimental input data from tier 2, modelling, kinetic models, probabilistic models accounting for uncertainties*) • n-PEC $_{sec\ comp}$ (*based on experimental input data from tier 2, modelling*)	

↓ ↓

Refined predicted environment risk quotient in initial compartment: n-PEC $_{ini\ refined}$ / n-PNEC$_{refined}$
Predicted environmental risk quotient in secondary compartment: n-PEC $_{sec\ comp}$/n-PNEC$_{sec\ comp}$

>1 -> Tier 3: refinement or risk mitigation

In case low durability is ascertained, i.e. the ENM rapidly loses ENM properties, the formed chemicals are handled as conventional chemicals. In case medium to high durability is stated, the first tier of the assessment scheme is entered. Any trigger value to differentiate between “high”, “medium” and “low” durability has to be discussed and agreed upon elsewhere.

Tier 1:

Once tier 1 is reached, a PEC-assessment for the initial compartment is to be performed. The resulting PEC is named as n-PEC $_{ini}$. The prefix “n“characterizes the PEC as a concentration assessed for “nanomaterials”. The suffix “ini” characterizes the PEC as a concentration in the initial compartment.

n-PEC_{ini} is determined on the basis of experimental environmental fate data, that means by using information on the stability as dispersion or emulsion, stability of the organic coating, modification of the ENM, e.g., by oxidation, dissolution / solubility rate, size, size distribution and shape.

For some of the mentioned endpoints new or modified test guidelines are needed. These are for example:

- solubility rate in the aquatic compartment (-> new test guideline needed),
- stability as dispersion or emulsion (-> new test guideline needed),
- stability of the organic coating (-> modified test guideline needed, e.g. on basis of OECD 301).

Furthermore, information on:

- size of the initial compartment,
- amount released into the initial compartment,
- and production volume

is needed as input data for appropriate default models.

Regardless of the risk quotient, a possible ENM transport to a secondary compartment – e.g. the transport from the aqueous phase to the sediment and transport within the sediment – needs further consideration on tier 2. The transport potential will be assessed on the basis of physico-chemical data, size, and size distribution rather than on complex fate tests.

Tier 2:

In case n-PEC ini / n-PNEC> 1 risk for the initial compartment cannot be excluded. A PEC-refinement on tier 2 is needed. The refinement requires further experimental fate data as input for a more sophisticated modelling.

Depending on the nature of the initial compartment (i.e., aquatic, terrestrial, sediment, sludge) the most important endpoints which should be considered are:

- (a)biotic transformation / degradation,
- mobility and transport in porous media; sorption / desorption to soil, sediment, and sludge; transformation in soil sediment, and sludge.

The more complex the considered environment matrix is the more environmental parameters interact with ENM which themselves are of complex nature. Consequently, the experimental test design needs to reflect this: The more complex the tested compartment is, the more should the experimental set-up mimic the real environment.

For tier 2 testing the following tests / test developments are recommended:

Table 22: Recommended fate testing for tier 2.

initial compartment	endpoint	test system	comments / recommendation
water	• stability in the aqueous phase, transformation / degradation • transport into sediment • sorption to sediment • transformation in sediment	• water sediment system (OECD 308 or modified)	Water sediment systems preferred over mesocosms which additionally include macrophytes and sediment inhabitants because of easier handling. Mesocosms might be too sophisticated and should be used on tier 3. The optimal size of the water / sediment system has to be agreed upon.
soil	• mobility and transport in porous media • sorption / desorption • transformation	• (aged) soil column leaching (OECD 312) • transformation in soil (modified OECD 307 or even new TG)	No further test on sorption / desorption (OECD 106 not appropriate) recommended but the use of OECD 312 (with additional guidance) instead. The appropriateness of OECD 307 on transformation in soil has to be proven and discussed; no experience available.
Sewage sludge	• sorption / desorption • transformation	• Sorption to sludge (new guideline needed) • OECD 303 A(modification to be discussed)	No appropriate test system to analyse sorption / desorption to sludge available but needs new development. OPPTS-guideline already is in the process of modification. Furthermore, it has to be checked whether OECD TG on transformation in sludge has to be modified

It is recommended to not only use pristine ENM for testing but also aged material in order to account for the durability in the respective compartment.

The tier 2 testing not only yields information on the fate in the initial compartment but also on the fate in a secondary compartment in case of transport. Secondary compartments are for example sediment (transport from the water phase), groundwater (transport through soil), and surface water (transport via surface run-off).

Based on the results from the tier 2 testing and the application of appropriate models n-PEC $_{\text{ini refined}}$ and n-PEC $_{\text{sec comp}}$ are assessed. Which models are appropriate models should be discussed elsewhere. This is beyond the objectives of this project.

As on tier 1 risk quotients (n-PEC $_{\text{ini refined}}$ / n-PNEC$_{\text{refined}}$; and n-PEC $_{\text{sec comp}}$ / n-PNEC$_{\text{sec comp}}$) are formed. In case the risk quotient is below 1 no further risk is expected and the tier procedure can stop. In case the quotient exceeds the value of 1 a further refinement should be made or risk mitigation strategies should be discussed (tier 3). Again, both is beyond the aims of this project and thus should be discussed elsewhere.

Models for PEC assessment do have to face the fact that environmental fate processes of ENM - which are dominantly aggregation, transformation and sedimentation processes - are non-equilibrium but kinetic processes. ENMs do not reach thermodynamic equilibrium but are present in the environment as unstable suspensions. Thus, conventional distribution models based on equilibrium processes such as the fugacity models developed by Mackay (Mackay, 1991; Mackay et al., 1992; Mackay, 2001) are not applicable. Few ENM fate models have been designed and evaluated which are capable to deal with the environmental complexity to predict realistic environmental concentrations of ENM (e.g., Praetorius et al., 2014; Sani-Kast et al., 2014). Such models are a highly valuable basis for further development and discussions.

Furthermore, it has to be discussed whether the use of probabilistic density functions is an appropriate tool to be further considered in PEC assessment. Such approaches (e.g. Gottschalk et al., 2009; Gottschalk et al., 2010) account for the fact that up to now much information on model input parameter is still lacking. These models are based on uncertainties.

7 Comparison of the test strategy with recommendation of German Competent Authorities and with the proposal RIP-oN2

The Fraunhofer-IME test strategy was compared with the proposals of the German Competent Authorities (German-Competent-Authorities, 2013) and with the outcome of RIP-oN2 (Hankin et al., 2011). These proposals are intended to give advice for the amendment of REACH and focus on specific aspects whereas the IME test strategy is more comprehensive. The German Competent Authorities focus their proposal on the strategy by linking production volumes of ENM and scope of testing. Specific properties of ENM are considered by preferring chronic tests instead of acute test and by consideration of agglomeration and sedimentation of ENM in the environment and therefore highlighting the testing of sediment organisms. The suitability of the procedures described in the TG is not taken into account. RIP-oN2 focussed on the suitability of the existing OECD-TG and addressed potential additional relevant specific intrinsic properties and endpoints. In Table 23 selected topics and parameter which are addressed in at least one of the three test strategies are compared.

Table 23: Comparison of selected topics addressed in the test strategies of Fraunhofer-IME, German-Competent-Authorities (2013) and RIP-oN2 (Hankin et al., 2011).

Topics	Fraunhofer-IME	German Competent Authorities	RIP-oN2
1. Scope of the strategy	Ecotoxicology	Characterisation of ENM, toxicology, ecotoxicology	Physico-chemical properties, toxicological endpoints, ecotoxicological endpoints
2. ENM	Consideration that ENM can have characteristics of "general" chemicals, but also of chemicals with active agent properties (for details see chapter 4.3) which are treated differentially within the different regulations	Pursuant to REACH established for chemicals	Not addressed
3. Test programme	Aquatic, sediment and terrestrial tests are applied equivalently	Stepwise process according to REACH (selection of test systems linked to production volume starting with aquatic tests followed by terrestrial tests on ENM with higher production volumes), but production volumes triggering the tests are lowered (e.g. tests to be performed for chemicals under REACH with production volumes >10 t have to be applied for ENM with production volumes >1 t). Exposure via sediment due to agglomeration and sedimentation has to be considered.	Not addressed
4. Chronic tests compared to acute tests	Chronic tests preferred	Chronic tests preferred.	Not addressed
5. Suitability of existing ecotoxicological TG	Not specifically investigated; suitability assumed due to various references	Need for guidance identified.	Considered as suitable with some limitations
6. New alternative endpoints	Currently, alternative endpoints to the conventional ones do not seem to be necessary	Does not exclude additional endpoints. If necessary, additional endpoints should be included in the assessment	No new additional relevant specific intrinsic properties are recommended for ecotoxicological information requirements.
7. Waiving criteria	Not excluded, but not specifically addressed	Specifically defined for ENM (e.g. poor water solubility alone cannot justify test waiving for ecotoxicity)	Not addressed

8. Potential candidates for new ecotoxicological endpoints	Various endpoints were addressed (among others the endpoints listed by RIP-oN2). The additional information provided by these endpoints was not considered convincing to include them in regulatory context and in the Fh-IME test strategy.	Not addressed	Fish ventilation rate, fish gill pathology, fish mucus secretion, fish brain pathology, animal behaviour, oxidative stress fish, Daphnia heart rate, Daphnia hopping frequency, number of cycles per minute of daphnia in appendage movement, Trojan horse effect of ENM
9. Bioaccumulation	To get information on the accumulation potential of ENM we recommend the determination of the ENM concentration in the test organisms of the ecotoxicological tests at the end of the incubation period.	Trigger based on lower production volumes (>10 t instead of >100 t) A fish-feeding study is to be given preference over the BCF test	Not addressed
10. Metric	Recommendation to present the results on basis of mass. In addition the surface area including the method used for the determination and the particle number should be mentioned.	Not addressed	No definitive conclusions on the best metric; sufficient characterisation of the forms of a substance to allow the dose-response to be expressed in the different metrics (number, surface area, mass).
11. Physico-chemical properties	Several physico-chemical data are needed on tier 1. Adaptation/modification of methods is recommended.	Additional physical-chemical properties need to be considered (morphology, size distribution, specific surface area, surface reactivity, surface charge, crystalinity).	Need for guidance on existing information requirements and test guidelines identified. Need for modification of TGs identified. Additional specific intrinsic properties identified (shape, surface area, porosity, surface energy, surface chemistry, surface acidity, surface charge, redox-potential, cell-free ROS/RNS production capacity).
12. Fate related properties	A tiered approach is recommended. Integrative test systems and simulation testing already recommended for the lower tier.	Tests of different complexity needed depending on tonnage. Adaptation of existing methods needed, however, the way of adaptation is to be phrased later. Simulation testing recommended for higher tiers.	Need for modifications of TG identified. Adsorption / desorption screening (TG modification needed) necessary for > 10 t/y products. No tiered testing scheme or simulation tests addressed.

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Part: Ecotoxicity

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