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# A Tiered Approach for Reliable Bioaccumulation Assessment of Manufactured Nanomaterials in the Environment Whilst Minimising the Use of Vertebrate Testing

## Scoping Review

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Environment Directorate

ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT

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[1] NanoHarmony – Towards harmonised test methods for nanomaterials. Grant agreement ID: 885931 is a co-ordination and support action co-funded by the European Commission research and innovation funding programme Horizon 2020.

[2] NanoSolveIT- Innovative Nanoinformatics models and tools: towards a Solid, verified and Integrated Approach to Predictive (eco) Toxicology, Grant agreement ID: 814572. Is a research project co-funded the European Commission research and innovation funding programme Horizon 2020.

[3] See https://www.oecd.org/chemicalsafety/nanomet/

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# Foreword

The OECD has an important role in standardising the methodologies used for the testing and assessment of chemicals. The Working Party on Manufactured Nanomaterials (WPMN) has been considering how best to proceed regarding manufactured nanomaterials (MNs) for more than a decade. This has included exploring a range of different MNs in existing tests, and asking if test methods need to be adapted to work better for MNs, or if new tests more relevant to the specific characteristics of MNs are needed. The WPMN also has a weather eye on the integration of testing so that overall testing strategies can give reliable and useful assessment of MNs. In this regard, there has been much effort on the physico-chemical properties and the toxicity of MNs. However, the aspect of bioaccumulation needs more attention.

Effort started on the topic of bioaccumulation during an OECD Expert Meeting on Ecotoxicity and Environmental Fate [ENV/JM/MONO(2014)1] hosted in Berlin in 2013. It was soon realised that the thinking needed to go beyond simply validating the existing fish bioaccumulation test for MNs. In fact, the entire logic for the bioaccumulation testing strategy needed some longer consideration. The idea of an integrated and tiered approach to bioaccumulation testing for MNs was proposed to the WPMN in 2014, with the UK leading and co-leads from Spain and Finland. At that time, existing data on the bioaccumulation of MNs in fish was very sparse, and it was also unclear if invertebrate bioaccumulation tests would work for MNs. The physico-chemistry of MNs with respect to bio-accessibility was poorly understood, but it was clear that the log $K_{ow}$ test was problematic as a 'chemical trigger' to initiate testing. A review paper discussed the ideas and possible approaches for a tiered approach to bioaccumulation testing was published (Handy et al., 2018).

Since 2018, Spain and others have made progress on collecting data for TG 305 with MNs, and the UK *via* data in EU projects such as NanoFASE (http://www.nanofase.eu/). However, the question remains on how best to build a testing strategy that leads to TG 305 and gives a clear understanding of the bioaccumulation potential of MNs. Given the plethora of potential MNs, there are concerns about workload and a desire to minimise vertebrate animal testing. This document sets out a preliminary tiered approach to bioaccumulation testing, the tools and techniques available, and how such an approach could be used to rationalise workload, screen out materials, and minimise the use of TG 305 with fish. It illustrates some of the possible alternative tests that could be included based on currently available evidence and provides recommendations for next steps for developing tools or guidance to support decision making in relation to TG 305.

# Executive Summary

The overall aim of this scoping review was to explore the possible options for a tiered approach to bioaccumulation testing, the available tools or test methods, and to provide data to show potential linkages between the possible tiers in the testing strategy, as well as the evidence-base for seeking alternatives to using live fish. An example scheme is outlined here, with four possible tiers to show the thinking on how such a scheme might work in practise. The tiers include: (i) chemistry triggers as alternatives to the log $K_{ow}$ test that are more relevant to the behaviour of nanomaterials (MNs); (ii) the inclusion of data from invertebrate tests, cell cultures, and/or *in silico* models to provide a weight of evidence for a bioaccumulation concern; (iii) an *in vitro* tier using fish gut tissue; and finally, tier (iv), the dietary method of TG 305.

Log $K_{ow}$ measures the lipid solubility of a substance and is often conducted to decide whether there is a need to proceed to the *in vivo* TG 305 test. The log $K_{ow}$ measurement was originally designed for soluble organic chemicals, and its use as a trigger for bioaccumulation testing strategies stems from the established notion that substances that are lipophilic also tend to bioaccumulate. However, the log $K_{ow}$ measurement is based on the steady-state thermodynamics of solutes, not the behaviour of MNs that exist as colloidal dispersions, and so the log $K_{ow}$ test may not work, or be inappropriate for many MNs. In such circumstances, it is currently necessary to proceed directly to *in vivo* bioaccumulation tests with fish. However, a new trigger(s) for testing, instead of the log $K_{ow}$, could be used that considers the physico-chemical properties of MNs. Primary particle size, hydrodynamic diameters of dispersions, particle settling, and dissolution are discussed as potential tools for a first tier in the testing strategy.

The use of vertebrate animal testing can be minimised by using a 'weight of evidence' approach in the testing strategy, that may include evidence from invertebrate bioaccumulation tests, *in silico* modelling, and/or existing data on fish or fish cell lines. There is evidence that invertebrate bioaccumulation tests work for MNs, and there is a strong ethical justification to use invertebrate tests instead of, or to minimise the use of, the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 305 on bioaccumulation in fish. In an aquatic scheme for MNs that settle from the water column, the OECD TG 315 for testing the bioaccumulation of new substances in sediment-dwelling benthic oligochaetes could be used to help a decision to waive or include TG 305, so that only the MNs of greatest concern are tested in fish. Briefly, in TG 315 worms are exposed to sediment spiked with the test substance, topped with reconstituted water, and the concentration of the test substance in/on the worms is monitored through exposure and elimination phases of the test. The uptake rate constant ($k_s$), the elimination rate constant ($k_e$) and the kinetic bioaccumulation factor ($BAF_K = k_s / k_e$) are then calculated. Alternatively, for MN that show a low settling rate, a bioaccumulation test with the freshwater amphipod, *Hyalella azteca*, could be applied which enables aqueous or dietary exposure of MNs and calculation of appropriate bioconcentration factors (BCF values). A draft OECD TG covering the *Hyalella azteca* bioconcentration Test (HYBIT) is currently under revision, but other aquatic invertebrate tests could be considered too. For example, conventions such as Oslo-Paris (OSPAR) do sometimes require data from marine organisms, and it may also be useful to develop a bioaccumulation test for MNs using marine bivalves or other marine species. The bioaccumulation of MNs in the terrestrial system should be considered. The earthworm test OECD TG 317 works well with MNs (e.g., CdTe quantum dots), and

therefore might have an immediate utility in a regulatory environment for MNs especially if bioaccumulation of MNs in the terrestrial system is assessed. Furthermore, earthworm data for MNs, so far, correlate well with fish data, and could be used to inform on whether to proceed to TG 305.

Traditional computational modelling techniques are being validated for MNs. *In silico* models that use particle metrics to predict the toxicity of MNs are also being developed, but these have not yet been widely applied to bioaccumulation. However, there are several computational tools that could be readily adapted to predict the bioaccumulation of MNs, and *in silico* approaches should be explored to aid decision making on whether a TG 305 test is needed for a particular MN in the scheme. This could include *in silico* read-across approaches to group MNs with similar properties and/or to compare MNs with the nearest bulk material where information on bioaccumulation may already be available. Data from fish cell lines could also form part of the weight of evidence to include or waive TG 305. Notably, the recently validated protocol for the RTgill-W1 cell line (TG 249) could be adapted to measure the bioaccumulation of MNs. Fish gut cells, such as the RTgutGC cell line, could be developed as assays to simulate dietary exposure.

From the perspective of animal welfare, and to minimise cross-species uncertainties, it may be desirable to include an '*in vitro*' fish tier in the testing strategy to inform on the need for any *in vivo* fish bioaccumulation test. For fish, dietary exposure is often more relevant for MNs than a water phase test. An *in chemico* digestibility assay is suggested as a possible tool that simulates digestion in the gut lumen of fish to determine bioaccessible fractions. This technique is rapid, taking only a few hours and does not use any animals. An *ex vivo* gut sac method from trout is also a rapid and useful tool for measuring the uptake of metal from MN exposures and could be further standardised for regulatory use. However, the latter uses 3-6 fish per treatment to collect gut tissue for replicates of the *ex vivo* preparation. Both tools, while well-established scientific methods for research on fish ecotoxicology and demonstrated with MNs, would need to be standardised and validated for regulatory applicability, also to agree how the endpoints could allow unambiguous assessment of 'bioaccumulative' or 'non-bioaccumulative' for MNs.

While suggestions for the possible tools and techniques that could be included in a tiered approach to bioaccumulation testing and assessment have been made, it is also important that any evidence from the early tiers in any such scheme are predictive of the bioaccumulation from MNs in fish *in vivo*. Such information will inform on the structure of any decision tree that determines how to move from one tier to another, and on how to exit the strategy. TG 305 is used to determine bioaccumulation (BCFs, waterborne exposures) or biomagnification factors (BMFs, dietary exposures) for substances in fish. While steady-state equilibria do not apply to MNs, a BMF-like approach is possible for MNs. Data sets from dietary exposures based on TG 305 in trout were explored in a meta-analysis approach (including Ag materials, CuO, and CdTe QDs with different coatings). Plots of total metal concentration in the tissue against ingested dose revealed an apparent (dynamic) steady state for the tissues of the rainbow trout (*Oncorhynchus mykiss*), such that nano biomagnification factors (*n*BMFs) could be calculated.

Similarly, in the terrestrial system nano accumulation factors (*n*BAF) could be calculated from accumulation curves for the earthworm, *Eisenia fetida*. There were strong correlations between the *n*BAFs in earthworms and the *n*BMFs in fish, indicating that earthworms could be very good predictors of bioaccumulation in fish. However, while this supports the notion of using invertebrate tests as an alternative tool to the TG 305 with fish, the adequacy of using a terrestrial test as a surrogate or predictor of an 'aquatic' test with fish needs to be explored.

The meta-analysis also explored the relationship between the physico-chemical properties of the MNs (primary size, hydrodynamic diameters, dissolution, settling rates) and the measured *n*BMFs in fish, or *n*BAFs of earthworms. Several particle metrics, on their own, gave reasonable correlations with the *n*BMFs in fish and *n*BAFs in earthworms, and although there was not one metric that best described all of the data, individual correlations with $r^2$ in excess of 90% were obtained. Multiple regression analysis

offers a simple and effective approach to predict *n*BMFs in fish from MN properties and could be used as part of the decision tree to inform on a bioaccumulation concern at the start of the testing strategy.

It was also possible to correlate inorganic/metallic '*in vitro*' fish data with the *in vivo* results of TG 305. The digestibility assay identified bioaccessible fractions of metals from MNs (CuO, Ag materials) that correlated with *n*BMFs in trout. Similarly, metal uptake in trout gut sacs also gave good correlations with the *in vivo* data. Both these techniques could be used to aid in a decision to conduct TG 305 when testing metallic nanomaterials. TG 305 using the dietary exposure method may be considered as the final tier. However, considering the 3Rs (replacement, reduction, refinement), the fish test should be avoided. Where possible, the one concentration approach (i.e., selected testing of one exposure concentration of the substance) should be used in TG 305 to minimise the use of animals within a test. However, this is only possible if concentration dependence of bioconcentration processes can be excluded.

There are knowledge/data gaps on some MNs. For example, data sets are needed more widely on carbon-based MNs with the digestibility assay and gut sacs. In general, the lack routine measurement methods for carbon-based MNs, including nanoplastics, carbon nanotubes and graphene oxide, have limited bioaccumulation testing with those materials. Computational tools to predict nanosafety have made advances but are not routinely applied to bioaccumulation data. A series of recommendations on the way forward and to close data gaps are made for the short, medium and long term.

# 1 Introduction

Determining the bioaccumulation potential is a key part of the environmental risk assessment of chemicals, including manufactured nanomaterials (MNs). Tests with vertebrate animals remain as part of the safety assessment of new chemicals. However, in keeping with the 3Rs (replacement, reduction, refinement) with respect to animal welfare, it is important that any testing is targeted, unnecessary testing is avoided, and that *in vivo* tests are used as a last resort. This also applies to bioaccumulation testing. The *in vivo* fish test for bioaccumulation potential, Test Guideline (TG) 305 (OECD, 2012), is often used for this purpose, with the option of either a waterborne or dietary exposure method, typically over 28 days. OECD Guidance Document 264 (OECD, 2017) also provides guidance on how to use the data obtained during a TG 305 test to calculate the bioaccumulation potential of a test substance, although the calculations are intended for traditional chemicals, and not yet validated for MNs (OECD, 2017). The dietary exposure method, especially, has found utility given the challenges of maintaining dispersions of MNs in water. Dietary exposure studies with fish have previously shown total metal accumulation from MNs such as $TiO_2$ (Ramsden et al., 2009), Ag (Clark et al., 2019a) and ZnO (Connolly et al., 2016). Moreover, with the development of detection methods such as single particle inductively coupled plasma mass spectrometry (spICP-MS) for fish tissue, it may be possible also to detect the particle number concentration in the fish from a MN exposure. From one such dietary exposure experiment, Clark et al. (2019b) confirmed the presence of Ag nanoparticles (NPs) inside the tissues of rainbow trout.

Measurement of the *n*-octanol-water partition coefficient (log $K_{ow}$) of non-nanoforms of a substance has frequently been used to determine whether bioaccumulation testing should be conducted. This assay measures the lipid solubility of the test substance and was originally devised with organic chemicals in mind; based on the notion that substances that tend to be lipophilic are also bioaccumulative in fish (Veith et al., 1979). The log $K_{ow}$ method relies on steady-state thermodynamic models, or other aspects of solute chemistry to predict bioaccumulation. Unfortunately, MNs form colloidal dispersions in liquids (i.e., they are not solutes) and the behaviour of the dispersion is dynamic (not steady-state equilibria). Therefore, the partitioning concept behind the log $K_{ow}$ test does not generally apply to MNs (Handy et al., 2012, Praetorius et al., 2014). Moreover, there are practical limitations to determine log $K_{ow}$ in MNs such as their tendency to form aggregates at the oil-water interface, which further strengthens the notion that log $K_{ow}$ may not be suitable to predict potential bioaccumulation properties of MNs.

Where use of log $K_{ow}$ is inappropriate, a 'weight of evidence' approach, as it has been applied for a metal bioaccumulation concern, may be used as an alternative. This evidence can be from regulatory relevant and reliable scientific literature, such as existing data on dietary bioaccumulation studies with fish, bioaccumulation studies on invertebrates, evidence from food web studies, calculated oral predicted no effect concentrations ($PNEC_{oral}$) in birds and mammals, as well as considering metal bioavailability in any bioaccumulation factor [e.g., (USEPA, 2007)]. However, guidance for dissolved metals may need further validation for use with metallic MNs. There may be incidental data from other standardised (eco)toxicological testing such as TG 408 for oral toxicity studies in mammals, if validated for MNs, that might be used to help provide a weight of evidence for bioaccumulation.

There is limited guidance from standardisation bodies, such as the OECD, on how TG 305 should be triggered or waived for MNs. The current default in the guidance if the log $K_{ow}$ cannot be determined for a soluble organic substance is to proceed directly to the *in vivo* fish test, TG 305. Similarly, in the absence of existing bioaccumulation studies on any metallic MN of concern to support a weight of evidence approach, then the default will be to conduct TG 305. This situation has unintended consequences for animal welfare, with potentially mandatory vertebrate animal testing in TG 305 for MNs. At present, although other invertebrate bioaccumulation tests exist, there are no agreed alternatives to vertebrate animal testing in the OECD scheme for bioaccumulation testing specifically in fish, and no mechanism to minimise the use of TG 305 for MNs. There is, therefore, a clear and urgent need to overhaul the bioaccumulation testing strategy for MNs, and recently a tiered approach to testing was proposed (Handy et al., 2018, Handy et al., 2021). This included four tiers: (i) chemistry triggers as alternatives to the log $K_{ow}$ test that are more relevant to the behaviour of MNs; (ii) the inclusion of data from *in silico* modelling, invertebrate tests, and/or cell cultures to provide a weight of evidence for a bioaccumulation concern; (iii) an *in vitro* tier using fish gut tissue; and finally tier (iv), the dietary method of TG 305 (Annex A, Figure A.1). The use of invertebrate bioaccumulation tests in the proposed tier 2 have also been discussed in detail (Annex A, Figures A.2 and A.3). Guidance in the risk assessment process for MNs has been provided for the use of bioaccumulation endpoints derived from studies using aquatic invertebrate species (Kuehr et al., 2021c). These scientific publications illustrate that an integrated and tiered approach to bioaccumulation testing is possible. The OECD community has built consensus on a possible way forward and how such a testing strategy could be designed (see Chapter 10).

# 2 Aims of the scoping review

The overall aim is to create a scoping document that considers a tiered approach to accurately determine the bioaccumulation potential of manufactured nanomaterials (MNs). The specific objectives include:

(i) a review of the possible methods and techniques that might be used as alternatives to testing fishes, exploring what tests are already available or under development;

(ii) providing evidence of correlations between possible lower tiers in any proposed scheme and TG 305 using existing data and some meta-analysis;

(iii) suggest suitable bioaccumulation endpoints derived from studies using alternative methods that could be applied in the risk assessment process for MNs;

(iv) provide the elements for a proposal of testing strategy;

(v) identify data gaps and what areas still need further investigation or development;

(vi) provide a series of recommendations that the Working Party on Manufactured Nanomaterials (WPMN) at the Organisation for Economic Cooperation and Development (OECD) can use to determine the next steps in moving this topic forward.

# 3 Benefits of waiving TG 305 in terms of animal welfare and the burden of testing

The knowledge of bioaccumulation potential in aquatic organisms is required under various global regulations, potentially resulting in the use of an extensive number of fish. There is also a concern, with the continued rapid growth of the nanotechnology sector, and with the rise in the development of new MNs for many applications, that the number of fish used for bioaccumulation tests will also significantly increase. However, a tiered approach to bioaccumulation testing offers an opportunity to develop a scheme that effectively addresses the bioaccumulation potential while minimising vertebrate testing.

There is a growing desire to ensure the 3Rs (replacement, reduction, and refinement of animals in research) are implemented and embedded within national and international legislation and regulations to ensure that experiments are only conducted in animals when scientifically justified. When animals are required, it is crucial that any tests use the fewest animals necessary to answer the scientific question and that appropriate animal welfare standards are maintained throughout. This also applies to the safety of chemicals including MNs. For example, under REACH it is stated that animals should only be used as a last resort and that alternative methods such as data sharing or read across approaches should be considered (e.g., https://echa.europa.eu/animal-testing-under-reach). Similarly, the US EPA has a programme aimed at reducing the use of vertebrate animals in the testing of chemicals (https://www.epa.gov/research/epa-new-approach-methods-efforts-reduce-use-vertebrate-animals-chemical-testing). The requirement for a TG 305 test, or similar with live fish, should be discouraged or waived, saving direct costs and other resources, as well as to improve animal welfare, whilst also achieving a scheme that is robust for chemical safety. For dissolved metals, if there is enough evidence from the literature that bioaccumulation is not a concern, then TG 305 might be waived. Similarly, for organic chemicals, there are existing opportunities to waive TG 305 if the log $K_{ow}$ is ≤ 3. Unfortunately, the equilibrium partitioning concept for log $K_{ow}$ is not applicable to many MNs (Praetorius et al., 2014). Nonetheless, alternative chemical triggers based on the physico-chemical properties of MNs (see below) may offer a mechanism to waive TG 305 and reduce the use of vertebrate animals. Furthermore, there are some scientific justifications to alter the testing strategy to move away from TG 305 for bioaccumulation testing (Handy et al., 2018). For example, MNs tend to agglomerate or aggregate in natural waters, leading to sedimentation, and consequently it may be more environmentally relevant to use a benthic invertebrate bioaccumulation test. Considering the ethical implication of TG 305, it should be avoided as a tool for predicting the bioaccumulation hazard of MNs in real ecosystems. The use of invertebrates as alternatives to fish are discussed below, along with other approaches such as *in silico* models. The ethical consideration is to use alternative methods for MNs to minimise the need for bioaccumulation testing with vertebrate animals.

If a TG 305 study is required, there are opportunities to reduce the numbers of fish used in each test for some substances. Historically, bioaccumulation studies have been carried out using three treatments (control, low- and high-dose exposure groups). In 2012, TG 305 was revised to recommend the use of

a single concentration – the 'one concentration' approach – for non-polar organic substances, reducing the number of fish used by a third. Bioconcentration factor (BCF) study reports for 55 plant protection products and 265 general chemicals (Burden et al., 2014, Creton et al., 2013), demonstrated that BCF values did not differ significantly between the high or low concentrations, thus providing support for the one concentration approach. However, the concentration independence for MNs is not yet proven in this context, and the concern is that like some dissolved metals, the BCF may be concentration-dependent for metallic MNs. Ideally, if only a single exposure concentration is tested for a MN, this concentration should be sufficient to enable detection in the tissue (i.e., avoid a false negative) and/or mimic environmentally expected concentrations, so that BCF is environmentally relevant, even if it is not yet proven to be concentration-independent for the given MN. Differing regional requirements in the data needed for regulation (Burden et al., 2016) may also limit the use of the one concentration approach at this time for MNs, although it is gaining acceptance for some chemicals [e.g., for pesticides (USEPA, 2020)]. The one concentration approach remains optional in TG 305, and currently needs some caution for MNs. However, once a body of data on the dose-effect of MNs has been obtained, then it may also be possible to adopt this approach for MNs to refine TG 305 testing where it cannot currently be eliminated.

## Agreeing quality criteria for scientific reports on bioaccumulation

To further implement the 3Rs, and avoid unnecessary testing, it may be that regulatory requirements or recommendations for new TG 305 testing could be waived by weight of evidence from the scientific literature. Any data mining from the scientific literature should be systematic, and with set quality criteria on the scientific papers used (Klimisch et al., 1997, Kase et al., 2016). In general, studies with a Klimisch score of 1 ("reliable without restriction") or two ("reliable with restriction") can be applied to regulatory toxicology. Studies with scores of 3 ("not reliable") or 4 ("not assignable") would not be suitable. The approach to data collection for this scoping review was in the spirit of the Klimisch score (Klimisch et al., 1997), but with additional criteria for particle characterisation. These additional criteria were: (i) appropriate particle characterisation through measurements of primary particle size and/or hydrodynamic diameter; (ii) measured metal concentrations in the test media that confirmed a consistent exposure and within 80% of the nominal concentrations; (iii) measured metal concentrations in the test organism were reported, and were detectable given the variation of background concentrations of metals in the control animals; (iv) evidence of quality assurance in the procedures for metal analysis, such as procedural blanks, spike recoveries, analysis of certified reference materials and internal standards to check for instrument drift over the course of the analysis; (v) the experimental design was replicated, at least $n$ = 3 tanks *per* treatment; and (vi) the experimental design had unexposed controls, and metal salt controls or bulk material controls as appropriate for a MN study design.

These criteria are rather stringent for the purposes of obtaining data by literature review, and while they may select scientific papers of good quality, there is the prospect of 'excluding' data that may be useful, especially when knowledge on the bioaccumulation of MNs is at an early stage. Some information on the particle size and its chemical composition is needed to identify the MN correctly, and most studies report primary particle size and/or hydrodynamic diameters. The measured total metal concentrations in the media and organism are needed to calculate bioaccumulation factors for the metals in MNs, and when validated detection methods become routinely available, measuring the nano form in both the media and the organism is desirable. Methods for measuring organic MNs in tissues are not readily available. At this time, the requirements on quality assurance in the analytical chemistry could be less stringent. For example, while procedural blanks and spike recoveries are possible to perform for some MNs, there are, as yet, no agreed certified reference samples for fish tissue containing known amounts of MNs that could be used to validate a measured tissue concentration for MNs following TG 305 or other bioaccumulation tests. So, this latter aspect is not practical to include as a criterion at present. The

criteria could be lenient on the inclusion of metal salt or bulk material controls in a MN study, because these are not needed to identify the bioaccumulation potential of the MN *per se*. Clearly, there are quality criteria for extracting data from the literature that could be used (Klimisch et al., 1997), with adaptations, to MNs. Data from the literature is one part of a wider weight of evidence approach that could help to waive or minimise the use of TG 305.

# 4 Methodology and meta-analysis

In addition to systematic literature review, the approach here was also to show the operation of the possible tiers in the testing strategy by meta-analysis of existing data, with the intention that the meta-analysis could be part of the evidence-base for agreeing how the integrated testing should be structured and to move through any tiers that are included. Full details of the meta-analysis approach are reported in Handy et al. (2021) and (Handy et al., 2022). The data used for the meta-analysis, so far, originated from primary experimental data sets collected at the University of Plymouth during the following EU projects: Sustainable Nanotechnologies (SUN, http://www.sun-fp7.eu/), NANOSOLUTIONS (https://nanosolutionsfp7.com/) and NanoFASE (http://nanofase.eu/). This included detailed information on the physico-chemical characterisation of the MNs with data on dissolution for all the materials in NANOSOLUTIONS (Vassallo et al., 2018), the CuO NPs used in NANOSOLUTIONS (Tatsi et al., 2018) and SUN (Boyle et al., 2020), and the Ag NPs and $Ag_2S$ NPs used in NanoFASE (Clark et al., 2019c). Part of this characterisation information is presented in Table 4.1, as extracted from Handy et al. (2021) and (Handy et al., 2022); and includes details of purity and primary particle sizes, as well as data about the particles dispersion and settling in ultrapure water.

For *in vivo* studies on rainbow trout, following TG 305 with additional measurements and replication, data was collected from the following studies: CuO NPs (Boyle et al., 2021); Ag and $Ag_2S$ NPs (Clark et al., 2019a). *In chemico* digestibility data for trout gut lumen were obtained from Handy et al. (2018) and for trout gut sacs from Clark et al. (2019c) for the Ag materials in the NanoFASE project; further unpublished data on other materials in gut sacs generated by Clark and Handy is included here using the same methodology. Raw data on total metal accumulation from MN exposures of earthworms were obtained from the following studies: CuO NPs (Tatsi et al., 2018); CdTe quantum dots (QDs) (Tatsi et al., 2020); and Ag and $Ag_2S$ NPs (Baccaro et al., 2018). Only metal-containing MNs were considered where total metal could be measured in the tissues of organisms by inductively coupled plasma optical emission spectroscopy (ICP-OES) or ICP-MS in order to inform on bioaccumulation. Carbon-based MNs such as single walled carbon nanotubes (SWCNTs) were not considered because of the absence of routine methods of measuring the uptake of such materials in fish and invertebrates, although dietary exposures based on TG 305 with trout had been previously conducted to assess toxic effects of $C_{60}$ and SWCNTs (Fraser et al., 2011).

## Table 4.1. Test materials physico-chemical characterisation

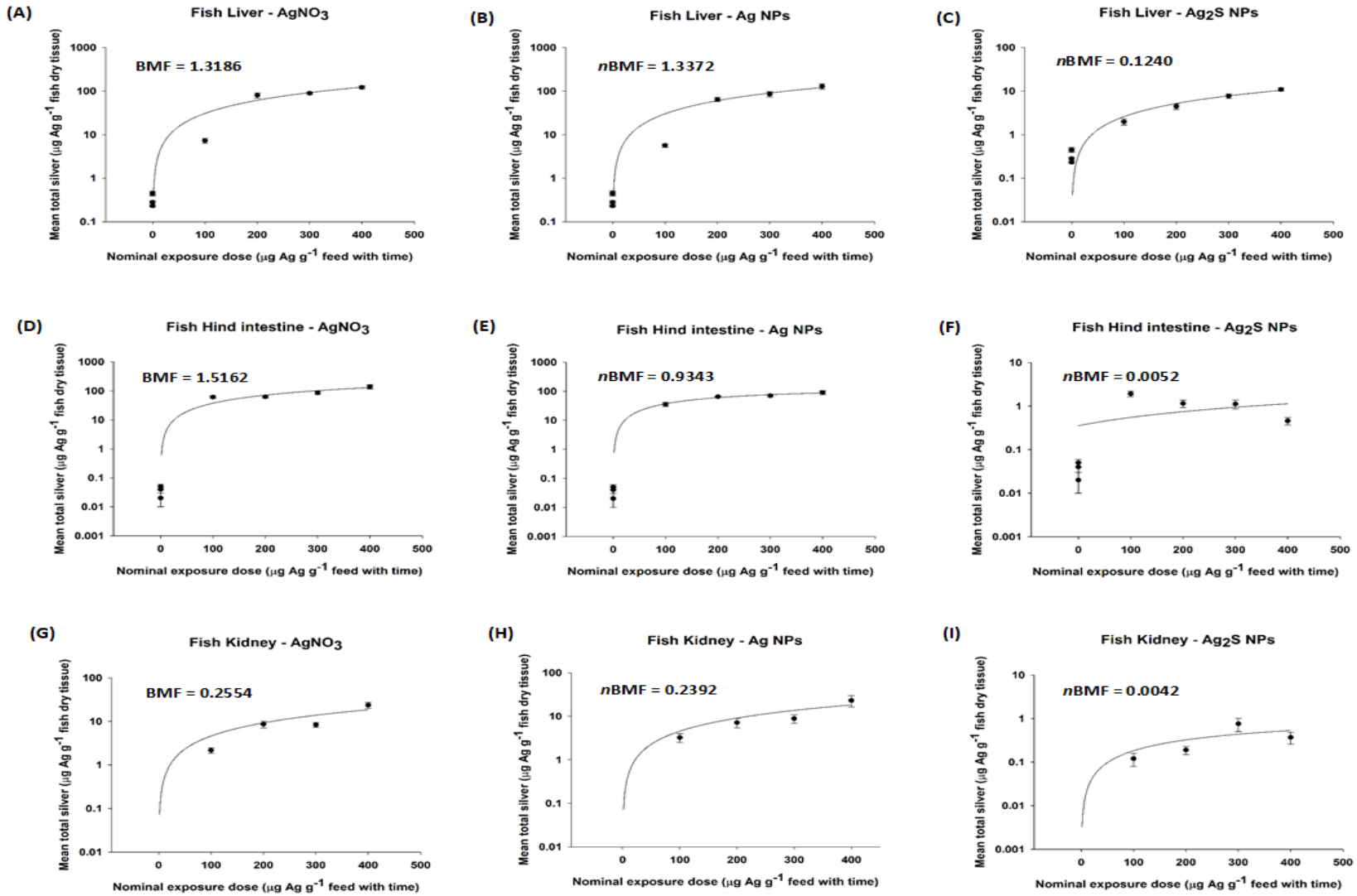
| Material | Manufacturer's information | [d] Primary particle size (nm) | [e] Hydrodynamic diameter (nm) | [f] Metal dissolution rate (µg $min^{-1}$) | [g] Settling rate in ultrapure water (mg $min^{-1}$) |
|---|---|---|---|---|---|
| [a] Ag NPs | Diameter, 50 nm; concentration 10.4 g $L^{-1}$ | 55 ± 3 | 66 ± 4 | 0.03 | ND |
| [a] $Ag_2S$ NPs | Diameter, 20 nm; concentration 9.6 g $L^{-1}$ | 37 ± 19 | 135 ± 7 | 0.00 | ND |
| $AgNO_3$, Sigma-Aldrich | Purity, > 99.00% | NA | NA | -- | NA |
| CuO Bulk, British Drug Houses Ltd | Analar grade | ND | ND | ND | 0.344 |
| [b] CuO NPs uncoated | Diameter, 10 - 20 nm; [c] surface area, 42 ± 2 $m^2$ $g^{-1}$ | 12.00 ± 0.37 | 41 ± 28 | 0.028 | 0.152 |
| [b] CuO NPs COOH-coated | Diameter, 10 - 20 nm; [c] surface area, 7.4 ± 0.5 $m^2$ $g^{-1}$ | 6.45 ± 0.16 | 121 ± 91 | 1.152 | 0.016 |
| [b] CuO NPs $NH_4^+$-coated | Diameter, 10 - 20 nm; [c] surface area, 6.1 ± 0.5 $m^2$ $g^{-1}$ | 9.53 ± 0.22 | 46 ± 36 | 0.31 | 0.043 |
| [b] CuO NPs PEG-coated | Diameter, 10 - 20 nm | 7.46 ± 0.42 | 100 ± 36 | 0.867 | 0.000 |
| $CuSO_4.5H_2O$, Sigma-Aldrich | Purity, 99.00 – 102.00% | NA | NA | ND | NA |

Note: [a] Supplied by Applied Nanoparticles (Barcelona) as part of the EU NanoFASE project. [b] Supplied by PlasmaChem GmbH as dry powder, with bespoke design as part of the EU NANOSOLUTIONS project. [c] Brunauer–Emmett–Teller (BET) surface area values (mean ± one standard deviation, *n* = 3) from EU NANOSOLUTIONS project. [d] Unless, otherwise stated based on transmission electron microscopy (TEM) images of material stocks in ultrapure water (18.2 MΩ, ELGA, UK) with data as mean ± standard error of the mean (S.E.M) and *n* = ≥ 60 measurements at University of Plymouth. [e] Particle size distribution measurements (mean ± one standard deviation, *n* = 3) by nanoparticle tracking analysis (NTA) on the material stocks in ultrapure water at University of Plymouth. [f] Maximum slope from rectangular hyperbola function of curve fit of the metal rate of dissolution from the material stocks in ultrapure water during dialysis experiments (*n* = 3) at University of Plymouth. [g] Maximum particle settling calculated from an exponential decay curve fit of the material stocks in ultrapure water (calibration curves *n* = 3) at University of Plymouth. NA - Data not applicable to the test material. ND - Not determined.
Source: Handy et al. (2021) and (Handy et al., 2022).

# 5 Particle properties and alternative chemistry triggers to the log $K_{ow}$ test

Given the concern that the octanol-water partition coefficient test, the log $K_{ow}$ concept, is not appropriate to the behaviour of particulates, and does not work for many MNs, an alternative chemical trigger is needed to start the overall testing strategy. The purpose here was to show if aspects of the physico-chemical properties of MNs could be used as predictors of bioaccumulation potential in fish. For example, if particle size, dissolution rate, or settling rate could predict bioaccumulation, and therefore act as a trigger for a decision to initiate testing. Previous work advocated using a dietary exposure method for fish in TG 305 (Handy et al., 2018) in order to best control the exposure for the determination of the bioaccumulation potential. The OECD defines dietary biomagnification factors (BMFs): '*as the ratio of the concentration of a substance in an organism to that in the organism's food at steady state*' (OECD, 2012). While MNs do not follow the steady-state equilibria expected of solutes between the external media and the tissue of the animal, it was still possible to measure the ratio of total metal concentrations in the food and tissues of rainbow trout exposed to metallic MNs *via* the diet to determine an apparent tissue specific nano BMF (*n*BMF). This was followed by plotting the nominal dietary dose against the measured total metal concentration in the fish tissue. The illustration for silver containing MNs exposures for trout *in vivo* are presented (Figure 5.1.). The nominal dose on the *x*-axis equals the concentration of the metal in the food from the nanomaterial multiplied by the exposure time (i.e., dose = concentration x time). For all the silver-containing substances, the liver and kidney showed a plateau in net metal accumulation, as expected for a non-essential toxic element that is not easily excreted (Clark et al., 2019a). In TG 305 the BMF is normally determined using test substance analysis of whole fish (wet weight basis). In this case, removal and separate analysis of the gastrointestinal tract may be employed to determine the contribution to whole fish concentrations for sample points at the end of the uptake phase and near the beginning of the depuration phase, or as part of a mass balance approach.

**Figure 5.1. Nominal feeding dose of silver as AgNO3, Ag NPs and Ag2S NPs, plotted against measured total Ag concentration in rainbow trout (*Oncorhynchus mykiss*) liver (A, B, C), hind intestine (D, E, F) and kidney (G, H, I).**



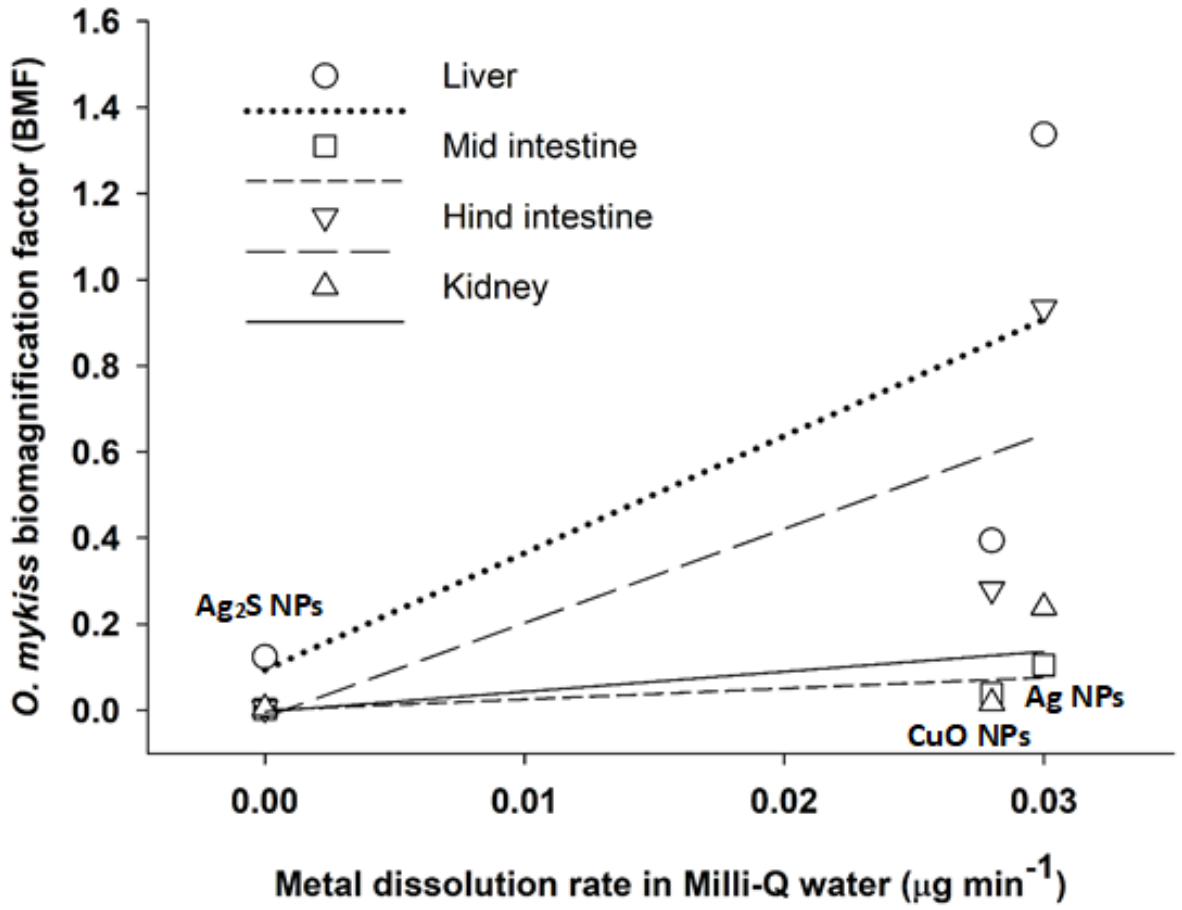
Note: Data are mean ± S.E.M, n = 4 – 6. Nominal feeding dose is calculated from the nominal concentration in the food multiplied by exposure time. Animals were fed a fixed ration size of 2% body weight per day.
Source: Clark et al. (2019a) and reported in (Handy et al., 2022)

With respect to particle metrics, the measured total metal concentration in the fish tissue at the end of the experiment was plotted against the physico-chemical properties of the MNs, such as the primary particle size, hydrodynamic diameter in water, and the metal dissolution rate in water (Handy et al., 2022). However, for regulatory use, the main interest was to then plot these particle metrics against the measured *n*BMFs for rainbow trout organs *in vivo*. For example, dissolution data were plotted against calculated *n*BMFs for the fish liver, mid intestine, hind intestine and kidney, respectively (Figure 5.2.), after exposure to the test materials CuO NPs, Ag NPs and $Ag_2S$ NPs. The liver and the intestines showed positive correlations, with dissolution explaining around half or two thirds of the *n*BMF, sometimes less (Figure 5.2). Similarly, if the nBMFs are plotted against primary particle size alone, or hydrodynamic diameter alone (any metric alone), then the single metric only partially explains the data. In other words, there is no single particle metric that fully predicts bioaccumulation in fish (Handy et al., 2022). However, this is no surprise, and if multiple regression is used with several particle properties together, much better predictions are obtained with $r^2$ values around 90% (Handy et al., 2022). This shows, in principle, that the approach is promising for regulatory use. However, regulations may require predictions with very high certainty (e.g., $r^2$ values above 95%). To increase the certainty in the correlations, more data should be collected for different MNs using trout in TG 305 to expand the meta-analysis, and this effort is current ongoing in the NanoHarmony project. Also using multiple regression of several particle parameters to derive the best possible predictions.

Other particle metrics should also be explored. Notably, the attachment efficiency, or alpha (α), for particle-particle interactions within a dispersion. The α represents (experimentally) the net effects of several particle properties that lead to particle settling according to Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Praetorius et al., 2014). For example, rather than trying to make correlations of bioaccumulation with lots of different individual properties of the dispersion, such as the particle number concentration, the size of the particles, their surface charge, other forces relating to repulsion or attraction, etc. It may be more practical to use an overarching descriptor such as α. Indeed, α, is much more relevant to MNs than the log $K_{ow}$ measurement (Praetorius et al., 2014) and can also be applied to heteroaggregation in natural waters (OECD, 2020b; Praetorius et al., 2020).

Substances are usually tested for environmental safety as they are produced (e.g., a pristine material from the manufacture). Here there is a concern regarding the environmental relevance of the testing strategy, and to ensure the nano forms present in real ecosystems are tested. It is expected that MN will age or be transformed by the surrounding environment. There are many possible transformations such as changes in oxidation state, dissolution, degradation of any coating on the surface of the MN, and the adsorption of natural organic matter, proteins, or other macromolecules to modify the surface of the material, as well as homo- or hetero-agglomeration that might affect bioavailability, and therefore subsequently the bioaccumulation of transformed MN (Lead et al., 2018, Spurgeon et al., 2020). Whether or not aged MNs bioaccumulate more than pristine MNs is unclear. In earthworms, aged MNs can show less [e.g., CuO, (Tatsi et al., 2018)], or the same metal accumulation [e.g., CdTe QDs, (Tatsi et al., 2020)] from MN exposure compared to the pristine form. The type of environmental matrix may also transform the MN to alter bioavailability and accumulation potential. For example, the bioavailability of silver from wastewater borne Ag NPs in the rainbow trout was investigated (Zeumer et al., 2020). The study showed that the bioavailability of Ag NPs (NM 300K) in fish was clearly reduced following wastewater treatment. Consensus on how to trigger testing for an 'aged' MN will need to be agreed, but for example, if the initial chemical triggers here show a significant difference between the pristine and aged MNs, then the aged MN might be considered as an additional target for bioaccumulation testing.

**Figure 5.2. Metal dissolution rate in water plotted against organ biomagnification factors for rainbow trout (*Oncorhynchus mykiss*) liver, mid intestine, hind intestine and kidney.**




Note: Data are mean ± S.E.M, n = 4 – 6. Organ total metal concentration was measured after 4 weeks exposure to either uncoated CuO NPs, Ag2S NPs or Ag NPs. Line of best fit, with the r2 values for liver, mid intestine, hind intestine and kidney are 0.51, 0.67, 0.59 and 0.35. Equations for the liver, mid intestine, hind intestine and kidney are y = 0.0933 + 27.1619x, y = 0.0003 + 2.5198x, y = -0.0158 + 21.8548x and y = -0.0032 + 4.6501x, respectively.
Source: (Handy et al., 2022).

# 6 *In silico* computational tools for predicting the bioaccumulation of MNs

The notion of using computational methods to relate the structural or chemical properties of substances to their hazards to make useful prediction tools is well-known for traditional chemicals. For many groups of chemicals, validated quantitative structure and activity relationship (QSAR) models are also available to aid in the prediction of bioaccumulation potential (ECHA, 2019). One possibility in the testing strategy could be to include such tools for MNs in one of the early tiers in order to help build a weight of evidence that a specific MN may be a concern, or not, with respect to bioaccumulation. However, the adaptation of QSAR models, or other *in silico* models, to MNs is at a relatively early stage in terms of validation for MNs and/or regulatory acceptance (Handy et al., 2018, Utembe et al., 2018). With respect to bioaccumulation, this is a data gap, and further work is needed to model physico-chemical properties of MNs to achieve *in silico* prediction tools that work across a range of MNs and their bulk or metal salt counterparts. Significant progress has been made in the development of a range of computational descriptors that can extend the information about the MNs, from image analysis, periodic table descriptors and more (as shown in Table 6.1). Such approaches are already being utilised to correlate with cytotoxicity, for example Papadiamantis et al. (2020a), and could also be extended to prediction of MN attachment, uptake and bioaccumulation.

Similarly, models for prediction of MN physico-chemical endpoints such as zeta potential which may change with the composition of the media, corona formation, etc., are emerging (Varsou et al., 2020, Papadiamantis et al., 2021), extrapolating from experimental data related to compositionally similar MNs to fill data gaps (Papadiamantis et al., 2021), and fully computational, utilising only periodic table and computational parameters (Varsou et al., 2020). Extensive work is currently underway to calculate these parameters and enrich the existing datasets related to fish bioaccumulation (from University of Plymouth) with these computational descriptors as a basis for the identification of the MNs descriptors most strongly correlated with bioaccumulation. Additionally, extension of the models to prediction of bioaccumulation and consideration of machine learning algorithms to unveil possible nonlinear relationships between the important MNs descriptors and bioaccumulation related endpoints is being implemented.

Computational approaches can take advantage of existing data to develop predictive nanoinformatics models, which can be used to either design MNs with specific properties (Melagraki and Afantitis, 2015, Varsou et al., 2019a, Ling et al., 2018, Martin et al., 2019) or to predict their behaviour and effects (Afantitis et al., 2018, Toropov et al., 2016, Basei et al., 2019). The integration of Machine Learning and the combination with custom-made solutions, such as implementation of read-across methods, is possible within NanoCommons and NanoSolveIT Cloud Platforms for Hazards and RA problems. The Cloud Platforms are based on web semantic cheminformatics and nanoinformatics application, with the ability to host any predictive model as a web service with a user-friendly user interface. Web applications, and similar computational tools, are invaluable for computer-aided design of MNs since

they can be used to predict the activity of new MNs prior to synthesis (i.e., safe by design with respect to adverse biological effects).

**Table 6.1. Summary of the different types of descriptors identified in the NanoSolveIT review that could be use *in silico***

| Descriptor Type | Examples | Number of descriptors reported to date | References |
|---|---|---|---|
| Descriptors from first principles-based calculations | | | |
| Quantum-mechanical descriptors (QM descriptors) | Standard heat of formation (HOF)<br>HOMO / LUMO | 12 | (Puzyn et al., 2011) |
| Periodic-table-based descriptors | Number of electrons of active metal<br>Atomic weight of active metal | 37 | (Kar et al., 2014) |
| 2nd generation periodic table-based MN descriptors | Core environment of metal defined by the ratio of the number of core electrons to the number of valence electrons | 15 | (De et al., 2018) |
| SMILES-based optimal descriptors | | 5 | (Toropov et al., 2012) |
| Metal-ligand binding properties | The covalent index<br>the cation polarizing power | 2 | (Sizochenko et al., 2014, Sizochenko et al., 2015) |
| Descriptors for MNs physicochemical characterization | | | |
| Liquid drop model-based descriptors | The number of MNs in the analysed agglomerate<br>The number of surface elements | 5 | (Sizochenko et al., 2014, Sizochenko et al., 2015) |
| Full-particle descriptors | Average potential energy of atoms in nanoparticle [eV]<br>Lattice energy of nanoparticle divided by the diameter of nanoparticle [eV/Å] | 62 | (Xia et al., 2011) |
| Bio-nano interface descriptors | Hamaker constant NM-NM<br>Hamaker constants NM-AA | 9 | (Power et al., 2019, Walkey et al., 2014, Kamath et al., 2015) |
| Image-based descriptors | Boxivity<br>Circularity<br>Min / Max Feret's diameter | 35 | (Odziomek et al., 2017) |
| Toxicogenomics descriptors | Molecular descriptors and gene modifications | Large numbers | (Serra et al., 2019) |
| | **Total** | **152** | |

HOMO; highest occupied molecular orbital. LUMO; least unoccupied molecular orbital. SMILES; simplified molecular-input line-entry system. eV, electron volts. Å, angstroms. AA, amino acid.

NanoCommons and NanoSolveIT follow the OECD principles for the validation of their predictive models. For example, Melagraki and Afantitis (2015) developed, a predictive classification model based on OECD principles, for the toxicological assessment of iron oxide MNs with different cores, coatings and surface modifications based on a number of different properties including size, relaxivities (relating to molecular spin, or the speed of new equilibria to stabilise in solutions), zeta potential and type of coating. More recently, a predictive nanoinformatics model, validated according to the OECD principles, has been developed for the prediction of the protein binding and the cytotoxicity of functionalized multi-walled carbon nanotubes (Varsou et al., 2019b). The application of *in silico* approaches for the prediction of specific MN properties is emerging as an important step towards complete *in silico* hazard and risk assessment.

Deep neural networks and deep learning algorithms have received much attention in the machine learning community and increasingly many other scientific, technological, business, and medical spheres over the last few years. These methods are closely related to or incorporated into big data analysis, especially for large volumes of high-resolution images. Applications of deep learning in the nanosafety discipline are, so far, quite rare. Information on the development of deep learning models using a variety of architectures aiming at object recognition of image classification has been produced by the EU project NanoSolveIT[4]. This project also developed the infrastructure for the implementation of deep learning models as web services with user friendly graphical interfaces. More specifically the machine learning (ML) model for prediction of multi-generational toxicity of MN to *Daphnia* work builds upon one of the most extensive ecotoxicological datasets available, using state-of-the art artificial intelligence (AI) methodologies to address the problem of identifying the effects of exposure to coated or uncoated $TiO_2$, Ag, or $Ag_2S$ MNs under different experimental conditions on *Daphnia magna*, based solely on electronic images (Karatzas et al., 2020). The deep learning technology could be applied on electron microscopy observations or synchrotron X-rays for fluorescent MNs to estimate the bioaccumulation of MNs in environmental species including earthworms and fish.

In addition, because MNs are not thermodynamically stable, bioaccumulation of MNs in vertebrates or other environmental species does not reach steady state. It is rather a dynamic process which depends on the level and the duration of exposure, any environmental transformations that may change the concentration or properties of the original MN, the exposure route, but also the rates of eliminating the MN. This may lead to the derivation of dynamic accumulation factors. As indicated in van den Brink et al. (2019), physiologically based pharmacokinetic (PBPK) models and biodynamic models are very useful tools for modelling and simulating the process of bioaccumulation as a function of time under different exposure conditions. Provided that MN concentration versus time data are available, NanoSolveIT has the expertise to develop PBPK models and fit the available data into the differential equations using various approaches such as Markov Chain Monte Carlo (MCMC) or mathematical optimisation methods. NanoSolveIT can also provide the infrastructure to deploy and publish these models as web services, make them accessible through application programming interfaces (APIs) to facilitate their use by (other) computer programmes and graphical user interfaces (GUIs) to facilitate their use by humans and integrate them into risk assessment workflows by combining internal exposure and bioaccumulation predictions with hazard assessment tools.

[4] See https://nanosolveit.eu/

# 7 Using invertebrate bioaccumulation tests as an alternative to fish

One approach to help minimise the need for bioaccumulation testing in fish is to collect evidence of a 'bioaccumulation concern' from invertebrate species instead (Handy et al., 2021, Kuehr et al., 2021c, Handy et al., 2018). Ultimately, the processes of uptake, storage, metabolism and excretion that control the accumulation of substances in the whole body of invertebrates such as oligochaete worms, molluscs, amphipods or *Daphnia* species are fundamentally similar to fish. This is because most of the molecular machinery is conserved across species and the anatomical barriers offer similar functional physiology with respect to the accumulation of MNs. See the recent review of uptake of MN in the animal kingdom that deals with these cross-species aspects (van der Zande et al., 2020). There will be some uncertainties on the numerical values of extrapolation factors across species for bioaccumulation, but the challenge here is not different to other chemicals, and some attempts to correlate (for example) BAF values in earthworm with BMF values in fish for MNs are emerging (Handy et al., 2021).

## Bioaccumulation tests with the freshwater amphipod, *Hyalella azteca*

From the perspective of animal welfare and to enable utility for environmental risk assessment, it may be preferred to have data on aquatic invertebrates as an alternative to data from tests on fish (i.e., keeping with the theme of aquatic organisms for the risk assessment). An aquatic invertebrate test for bioaccumulation potential as alternative to, or for decision making on, fish tests would be a very useful tool. Notably, the use of the freshwater amphipod, *H. azteca*, has been advocated for the bioaccumulation testing of MNs in a non-sediment approach (Kuehr et al., 2021b).

The suitability of *H. azteca* as an alternative test organism for bioconcentration studies with organic compounds was investigated (Schlechtriem et al., 2019, Kosfeld et al., 2020). An international ring test has recently confirmed the high potential of the *H. azteca* bioconcentration test (HYBIT) to be used as non-vertebrate alternative for bioconcentration studies according to OECD TG 305. The transferability of the test protocols (semi-static and flow-through approach) as well as the repeatability of the results obtained were proven. A new OECD TG for *H. azteca* is under revision with the WNT commenting rounds being on the way. In addition to the broad range of organic compounds which have been tested so far, *H. azteca* has been shown to be suitable for testing MNs (Kuehr et al., 2021b) and the new test system may be thus of high value also for the regulatory assessment of MNs. In contrast to bioaccumulation tests with bivalves, Gastropoda, Branchiopoda, or earthworms, where BAF values can be calculated based on the combined waterborne and oral exposure of the organisms during the test, using *H. azteca* would allow derivation of distinct BCF or BMF values for aqueous and dietary routes respectively. A suitable flow-through system for MN exposure using Zuger glass jars or aquaria, and protocols for dietary exposure without the risk of uncontrolled co-exposure *via* the water are available (Kuehr et al., 2021b). Bioaccumulation test can be also carried out under semi-static conditions as long as the concentration of the test substance in the test chamber is maintained within ± 20% of the mean of the measured values during the uptake phase (OECD, 2012).

The pros and cons of using *H. azteca* for bioaccumulation testing were described in detail by Schlechtriem et al. (2019). Even if the organism is too small to allow a tissue or compartment-based analysis after dissection, there are protocols and methods available that may allow the localisation of MNs in the different tissues by imaging methods or measurements, like spICP-MS (Kuehr et al., 2020a, Kuehr et al., 2021b). This knowledge will also feed into the new OECD GD on the determination of MN concentrations in biological samples, which is under development within the OECD Test Guidelines Programme (https://www.oecd.org/chemicalsafety/testing/work-plan-test-guidelines.pdf). Studies have been conducted on $Al_2O_3$, CuO, ZnO, Au and Ag nanoparticles using *H. azteca* (Kuehr et al., 2021c). Considering the broad knowledge on the ecology, physiology, metal uptake process, and detoxifying mechanisms of amphipods, as well as the long-term experience obtained from metal bioaccumulation studies with amphipods and the growing experience with MNs, the benthic species *H. azteca* could play a key role in the bioaccumulation assessment of MNs. Further MNs with different characteristics (including organic and inorganic MNs) are currently being tested to extend the knowledge on MN bioaccumulation in freshwater amphipods, and to allow an improved assessment of the applicability domain of the *H. azteca* test within a tiered testing strategy for MNs.

The *H. azteca* bioaccumulation tests have been shown to lead to higher BCF/BMF values compared to fish as described for Ag NPs (Zeumer et al., 2020, Kuehr et al., 2021b). However, further MNs need to be tested and compared with MN data on fish bioaccumulation to assure the conservativeness of the alternative testing/model (invertebrate test as a worst-case approach). This would ideally lead to make decisions to waive a fish test according to OECD TG 305 also where a test on invertebrates, as part of a weight of evidence approach, shown not to be bioaccumulative.

## Bioaccumulation tests with other aquatic invertebrates

The use of aquatic invertebrates in a tiered approach to bioaccumulation testing has also been recently and independently reviewed (Kuehr et al., 2021c), showing that it is possible to measure the bioaccumulation of substances from MN exposures in further organisms such as bivalves, gastropods, and brachiopods. The bioaccumulation of MNs (Ag NPs and $TiO_2$ NPs) in the freshwater bivalve, *Corbicula fluminea*, was also investigated by (Kuehr et al., 2020b). In the case of metal-containing MNs, it is possible to measure the total metal in the organisms, or sometimes to measure metal isotopes [e.g., (Croteau et al., 2014)].

In addition to invertebrates that graze on sediments or substrate, such as aquatic snails, and other epibenthic invertebrates such as amphipods (e.g., *H. azteca*), some consideration should be given to invertebrates that burrow into freshwater or brackish water sediments (e.g., aquatic worms). OECD TG 315 describes a method to assess bioaccumulation of sediment-associated chemicals in endobenthic oligochaete worms. The test consists of two phases. During the uptake phase, worms are exposed to sediment spiked with the test substance, topped with reconstituted water and equilibrated as appropriate. Change of the concentration of the test substance in/on the worms is monitored throughout both phases of the test. The uptake rate constant ($k_s$), the elimination rate constant ($k_e$) and the kinetic bioaccumulation factor ($BAF_K = k_s / k_e$) are calculated. For the blackworm, *Lumbriculus variegatus*, exposed to Ag MNs in sediments with overlying dechlorinated tap water, it was possible to determine the total Ag uptake kinetics and estimate apparent bioaccumulation factors (Coleman et al., 2013). This species of worm also has utility for conducting bioaccumulation measurements in simple aqueous media without sediment. Khan et al. (2015) used a modified M7 media [without the metal chelator, ethylenediaminetetraacetic acid (EDTA)] to demonstrate the uptake kinetics of Ag MNs with different surface coatings; and derived the uptake and elimination rate constants for the MNs. *L. variegatus* has also been used for kinetic studies with radiolabelled carbon nanotubes (Petersen et al., 2008).

For activities that take place in marine environments, such as the oil and gas sector, there are approaches defined by OSPAR, for example, with regard to whole effluents where a tiered approach to testing is already adopted (OSPAR, 2007). This scheme uses physico-chemical tests as initial predictors of a bioaccumulation concern, solid phase microextraction (SPME) or liquid-liquid extraction (LLE), both of which rely on equilibrium solute chemistry and may therefore not be appropriate for MNs. Importantly, further bioaccumulation testing is indicated if a concern regarding bioaccumulation is raised. This could include conducting TG 305, but with a relevant marine species; or marine bioaccumulation tests on fish and bivalves following the American Society for Testing Materials (ASTM) methods [e.g., (ASTM, 2013)]. The log $K_{ow}$ measurement is also used for testing offshore chemicals, followed by bioaccumulation tests on filter feeding marine bivalves (e.g., mussels, clams or oysters) and recommended for 'substances that give rise to suspended particles' (OSPAR, 2020). These protocols from marine species are standardised, although not yet validated for MNs. Importantly, the OSPAR approach readily accepts marine invertebrate tests to replace, or complement, studies carried out with fish. This is similar to the thinking here for the tiered approach to testing with TG 305.

Marine bivalves have utility for bioaccumulation studies with MNs, partly because the exposure is relatively easy to manage for these filter feeders and the organs are big enough to dissect for the determination of the body distribution of the MN (Petersen et al., 2019). Marine invertebrates are known for biogenic particle formation as part of their normal metal homeostasis, and so background concentrations of MNs in the organisms may need to be established. For example, Gallocchio et al. (2020) demonstrated $TiO_2$ particles in the edible mussel, *Mytilus galloprovincialis*, following exposure to either $TiO_2$ MNs or ionic Ti using spICP-MS. The digestive gland of *Mytilus* species, as expected from metal homeostasis, is also a target organ for metal from exposure to metal-containing MNs [e.g., Ag MNs (Jimeno-Romero et al., 2017)] and the wealth of experience of bioaccumulation studies on metals in marine bivalves might be applied to MNs. Similarly, MNs may accumulate in the gills of bivalves [e.g., $TiO_2$ (Shi et al., 2019)], but they can also be largely excreted in the pseudo-faeces [e.g., $CeO_2$, (Conway et al., 2014); Au, Ag, (Kuehr et al., 2021a)]. Thus, bivalves show the expected features of both uptake and excretion of MNs.

Although bivalve molluscs have been the most used taxa in this context, other taxa should be considered. As noted above, *L. variegatus*, will tolerate saline conditions over sediment, and is used in the OECD TG 315 bioaccumulation test (OECD, 2008). Other species might be considered for marine bioaccumulation studies with MNs; such as the polychaete worm, *Arenicola marina*; the amphipod, *Corophium volutator*; and gastropod snails (*Littorina spp.*). Further review of the invertebrate literature with some meta-analysis may also reveal the reliability of read across from freshwater to marine species for MNs, as well as identifying the most sensitive marine invertebrate for bioaccumulation studies with MNs.

## Bioaccumulation tests with earthworms

Bioaccumulation testing is well established with earthworms and there is an internationally agreed protocol, OECD TG 317, for testing the bioaccumulation of new substances in earthworms, and enchytraeids which are smaller soil animals commonly called 'potworms' (OECD, 2010). The TG 317 consists of an uptake (exposure) phase and an elimination (post-exposure) phase. During the exposure phase, concentrations of the test substance are measured in the worms for up to 14 days (enchytraeids) or 21 days (earthworms) until 'steady-state' (for solutes, not MNs) is reached. During the elimination phase in clean soil, measurements are made at 14 days (enchytraeids) or 21 days (earthworms), unless earlier measurements show a 90% reduction of the test substance residues in worms, in which case the study may be concluded. The uptake and elimination data are then used to calculate the bioaccumulation factor. The earthworm test works well with MNs (e.g., CdTe quantum dots, (Tatsi et al., 2020) and therefore TG 317 might have an immediate utility in a regulatory environment for MNs.

For the earthworm bioaccumulation test, TG 317, like the fish bioaccumulation test, the concern was whether an apparent steady state could be achieved in order to validate any nano BAF (*n*BAF) approach. Details of the meta-analysis on earthworm are reported elsewhere for a range of MNs including CuO NPs, different forms of Ag NPs, and CdTe QDs (Handy et al., 2021), but it was possible to plot total metal concentrations in earthworms *versus* the exposure dose to identify where there was an apparent plateau in the accumulation curves. The latter was then used to estimate *n*BAFs for the earthworms. Peer-reviewed literature on the bioaccumulation of MNs in earthworms was collected (Handy et al., 2021) for the species *Eisenia fetida*, *Eisenia andrea* and *Lumbricus rubellus*. Part of this data is presented in Table 7.1. These studies provided enough information on the exposure, the properties of the MNs and the measured total metal concentrations in the animals, in order to calculate bioaccumulation factors (BAFs). The reported BAFs for earthworms, from these publications, are shown in Table 7.1. It appears that the BAF values may be size-dependent within Ag NPs, but so far, it is unclear if bulk forms of metal-containing materials would have different BAFs to the MN form.

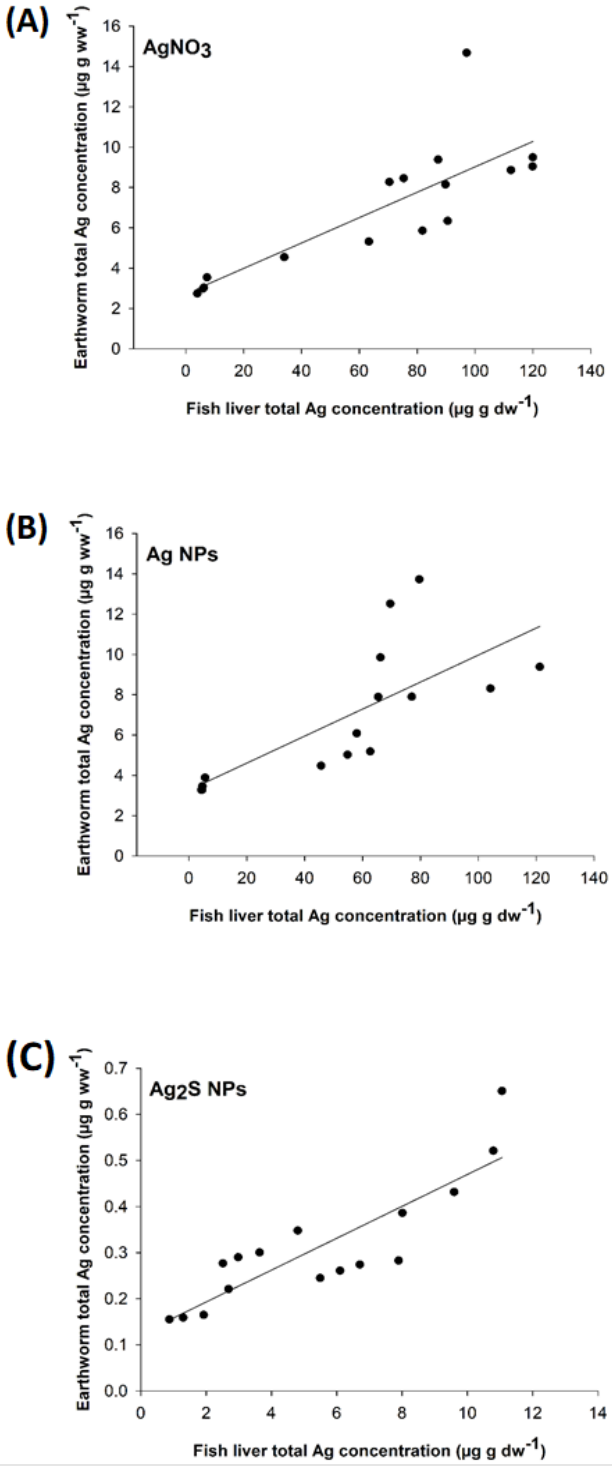
It would also be useful to know if the bioaccumulation in earthworms could be used to predict bioaccumulation in fish for exposures to MNs. Some example plots are shown in Figure 7.1 of correlations between the total metal concentrations from MN exposures in earthworms compared to fish tissues for the same materials. Following exposure to $AgNO_3$, Ag NPs or $Ag_2S$ NPs (Figure 7.1, A-C), there was a significant relationship (Pearson's correlation, $p < 0.01$) between the fish liver total silver concentration and the respective whole body earthworm total silver concentration. This result illustrates the notion that earthworms could be predictors of concentrations in fish (liver) as part of a testing strategy for Ag-containing MNs. The $r^2$ values for the fits indicate a good similarity for silver with values of 0.6702, 0.5306 and 0.7502 for the $AgNO_3$, Ag NPs and $Ag_2S$ NPs, respectively. Similar findings were obtained with other metallic MNs (Handy et al., 2021). It would also be beneficial to know if the prediction procedure would work for more than one metal, and if grouping approaches improved the resolution of the meta-analysis. In this regard, data from both Ag and Cu-containing MNs were combined, since the metals have similar valencies and reactivity inside animal tissue (i.e., intracellular $Cu^+$ and $Ag^+$). When the *n*BMF for fish organs were plotted against the *n*BAF of the earthworm, for both Cu and Ag together, the $r^2$ values for a linear fit were 0.99, 0.94, 0.89 and 0.97 for the liver, mid intestine, hind intestine and kidney, respectively (Handy et al., 2021). This shows that the prediction is stronger when metals are grouped by similar chemistries. The Pearson correlation values were also found to be significant ($p < 0.05$) for all the organs measured individually in fish (mid- and hind-intestine, liver, kidney) relative to the earthworm tissue.

## Table 7.1. Derived earthworm-soil bioaccumulation factors (BAFs) for silver in peer-reviewed literature

| Material | Primary particle size | Nominal exposure concentration (mg $kg^{-1}$) | Bioaccumulation factor[a] | Species | Method | Exposure duration | Authors |
|---|---|---|---|---|---|---|---|
| $AgNO_3$ | - | 100 | 0.05 | *Eisenia fetida* | (OECD, 2004) | 28 d | (Shoults-Wilson et al., 2011) |
| Ag NPs | 30 – 50 nm | | 0.01 – 0.02 | | | | |
| $AgNO_3$ | - | 15 | 7.8 | *E. fetida* | (OECD, 2010) | 28 d | (Baccaro et al., 2018) |
| Ag NPs | 50 nm | | 9.5 | | | | |
| $Ag_2S$ NPs | 20 nm | | 0.8 | | | | |
| $AgNO_3$ | - | 1.09 | 0.74 | *Eisenia andrei* | (OECD, 2010) | 21 d | (Velicogna et al., 2017) |
| Ag NPs | 20 nm | 3.9 | 0.89 | | | | |
| Ag NPs | 5 nm | 50 | 0.096 | *E. fetida* | (OECD, 2004) | 28 d | (Garcia-Velasco et al., 2016) |
| $AgNO_3$ | - | 15 | 0.033 | *Lumbricus rubellus* | (ISO, 1998) | 28 d | (Makama et al., 2016) |
| Ag NPs | 50 nm | 250 | 0.002 | | | | |
| $AgNO_3$ | - | 154 | 0.200 | *L. rubellus* | (ISO, 1998) | 28 d | (van der Ploeg et al., 2014) |
| Ag NPs | 15 nm | 15.4 | 0.023 | | | | |
| $AgNO_3$ | - | 200 | 0.030 | *E. andrei* | (OECD, 2004) | 28 d | (Schlich et al., 2013) |
| Ag NPs | 15 nm | | 0.060 | | | | |

Note [a] Bioaccumulation factor used as a general term with no specific distinction being made here between diet uptake and uptake from direct environmental contact. – Data not applicable to the test material.
Source: Part of Handy et al. (2021).

**Figure 7.1. Correlations for linear regressions between whole body total metal concentrations in earthworms and fish liver of the testing strategy originally proposed by Handy et al. (2018).**



Note: Data are for exposures to: (A) $AgNO_3$, (B) Ag NPs and (C) $Ag_2S$ NPs. The earthworms were exposed to soil containing nominally 100 mg Ag/kg for 28 days, and the fish were fed a diet containing nominally 100 mg Ag/kg for 28 days. The $r^2$ values were 0.6702, 0.5306 and 0.7502 for the $AgNO_3$, Ag NPs and $Ag_2S$ NPs, respectively. The equations of the line are (A) $y$ = 6e-05$x$ + 2.7308, (B) $y$ = 7e-05$x$ + 3.2678 and (C) $y$ = 3e-05$x$ + 0.1238. The correlations show that earthworm data could be predictive of bioaccumulation in fish and be used to help decide if a TG 305 fish test is needed for a given MN.
Source: The total Ag earthworm concentrations are from Baccaro et al. (2018) and the fish liver concentrations are from Clark et al. (2019b). Redrawn from (Handy et al., 2021).

Overall, this data shows that *n*BMFs in fish can be predicted from *n*BAFs in earthworms, with the latter derived using an existing OECD Test Guideline (TG 317). The correlations with fish liver were especially strong (99%) for metallic MNs and may even argue that the fish test may not be needed at all, when such good predictions of bioaccumulation can be measured in earthworms. In practice, there are currently limited established alternatives to vertebrate testing to satisfy regulatory requirements for bioaccumulation testing where sufficient historic data does not already exist. There is an opportunity now to strive for the use of invertebrate tests to waive or minimise the use of TG 305 on fish. Data on carbon-based and organic MNs would be needed to determine if this concept could be more widely applied to other compositions of MNs, but in principle for read-across the uptake mechanisms (e.g., endocytosis) are conserved across species. Clearly, bioaccumulation tests with earthworms result in (*n*)BAFs rather than BCF or BMF values and some agreement would be needed on methods for any extrapolation between these endpoints, and for thresholds to be used in decision making on bioaccumulation potential. Currently, *n*BAFs values are thus difficult to use for regulatory bioaccumulation assessment because clear threshold values are missing/not agreed. An alternative method which allows the estimation of established regulatory endpoints would be advantageous.

An alternative method which allows the estimation of established regulatory endpoints, such as BMFs, would be advantageous. A dietary exposure test system was developed to investigate the biomagnification of organic chemicals in the terrestrial isopod, *Porcellio scaber* (Kampe and Schlechtriem, 2016). Adult isopods were fed on alder leaf powder (*Alnus glutinosa*) spiked with the test substance. The method has uptake (16 d) and depuration phases (16 d) to allow the calculation of kinetic uptake and depuration rates, and therefore estimated kinetic biomagnification factors (BMFs). A slightly modified approach was applied to investigate the bioavailability of sulfidized Ag MNs to *P. scaber* (Kampe et al., 2018). Most of the total Ag measured in *P. scaber* at the end of the uptake phase was found in the hindgut (71%), indicating that only a minor part of the estimated Ag content was assimilated by the isopods, with 16.3 and 12.7% found in the carcass and hepatopancreas, respectively. Nonetheless, the test system could be used to estimate *n*BMF values.

# 8 *In vitro, in chemico* and *ex vivo* alternatives to *in vivo* fish tests

From an animal welfare perspective, it may be desirable to replace a fish test with *in vitro* methods using fish cells or tissue. These can also provide a rapid screening tool, so that only bioaccumulative materials of concern are tested *in vivo*. The next section considers a range of approaches including *in chemico* digestibility assays, the use of fish gut sacs, and fish cell culture.

## *In chemico* digestibility assays for bioaccessible fractions

The digestibility assay approach is a sequential simulation of digestion in the gut lumen of fish that can be used to determine the bioaccessible fractions of the test substance in each region of the gut. The notion here is that any bioaccessible fraction might be available for uptake, and therefore, enable bioaccumulation. This thinking is similar to that for human digestibility and the risks from contaminated foods (More et al., 2021). In terms of developing a standardised protocol that might be familiar to stakeholders, the approach is similar to the unified BARGE sequential extraction which is a standardised method for determining the bioaccessibility of chemicals in soil, and has also been applied to MNs (Vassallo et al., 2019). The protocol here is intended to mimic the gut lumen of a freshwater fish such as rainbow trout (Handy et al., 2018) for extraction from fish food pellets. Sequential extractions are performed in 0.9% NaCl, saline plus 10 mmol $L^{-1}$ (EDTA), and then in saline plus 0.1 M HCl, and finally a strong acid digestion in *aqua regia* for the remaining total metal. The interest here is whether the digestibility assay is a good predictor of bioaccumulation *in vivo*, or at least uptake to an internal organ.

## Using gut sacs to determine uptake *ex vivo* and predict bioaccumulation potential in fish

The use of gut sacs from fish was suggested recently for MNs (Handy et al., 2018). The gut sac method uses fresh tissue collected from a fish, and to test a MN with appropriate controls with replicates, may use five or six fish in total; but this is still far less than TG 305 (typically using 150 fish). This approach uses the gastrointestinal tract of a fish, cut into the relevant anatomical sections, and with the gut lumen filled with the MN of interest in physiological saline, and then incubated for 4 hours. The uptake of total metal from metallic MNs (for example) is then measured in the mucosa, underlying muscularis and serosal (blood side) compartment, and then compared against a relevant bulk material or metal salt control. The method is quick (easily prepared and completed in one day), using simple laboratory equipment and is inexpensive to conduct and does not require sterile conditions. The gut sac approach has a long history in both physiology (Wilson and Wiseman, 1954) and the uptake of chemicals (Handy

et al., 2000) and also for MNs in fish ($TiO_2$, (Al-Jubory and Handy, 2013); Ag NPs, (Clark et al., 2019c); CuO NPs, (Boyle et al., 2020).

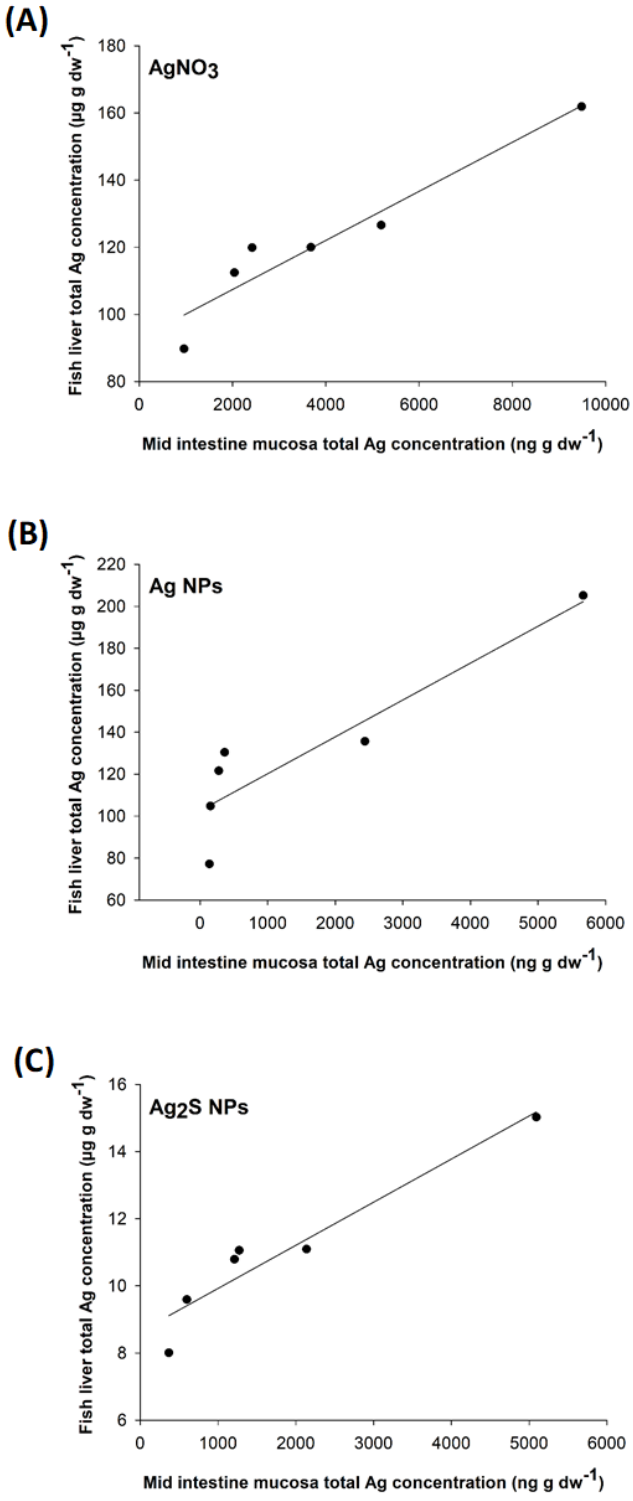
Figure 8.1 shows an example for Ag-containing materials where the gut sac experiments were performed using the routine method by Clark et al. (2019c). The total metal concentrations in the mucosa of tissue from the gut sac experiments were then plotted against that of the liver from *in vivo* experiment with the same substances: dissolved Ag, Ag NPs, and $Ag_2S$ NP (Figure 8.1.). There were statistically significant positive correlations (Spearman's or Pearson's correlation, $p < 0.01$) between the gut sac result and *in vivo* fish data. For the Ag materials, the $r^2$ values of the correlations were 0.9167, 0.8326 and 0.9142 for the $AgNO_3$, Ag NPs and $Ag_2S$ NP treatments, respectively. This is arguably, a strong correlation between *in vitro* and *in vivo*, and in the case of the Ag-containing MNs, both the alternative methods used here correctly predicted a bioaccumulation concern *in vivo*. It is possible to calculate BMF values for different anatomical regions of the gut from the gut sac studies (Handy et al., 2022). For instance, the $AgNO_3$, Ag NPs or $Ag_2S$ NPs BMF or *n*BMF values for the mid intestine were 0.231 ± 0.110, 0.072 ± 0.101 and 1.027 ± 1.096, respectively. When these were compared to *in vivo* BMF values, both the mid intestine and hind intestine produced strong negative correlations, with $r^2$ values of 0.9821 and 0.7943, respectively (Handy et al., 2022). The gut sac method also offers a reduction in the use of animals. The gut sac method has clear utility for the metallic MNs published above, and with further data coming from the NanoHarmony project on Cd/Te quantum dots, ZnO and $CeO_2$ materials. This could be considered as a tool to help waive or include TG 305 in the testing strategy.

## Use of fish cell cultures to predict bioaccumulation potential

Fish cell lines are an obvious alternative to *in vivo* work with fish, with the broadest range of cell lines being available from rainbow trout (Bols et al., 2017). Accumulation of total Ag from Ag NP exposures was quantified in detail in the rainbow trout gill cell line, RTgill-W1. Rapid particle uptake was also unequivocally demonstrated by electron microscopy; and the total Ag accumulation occurred in a time and concentration-dependent manner with near apparent steady-state concentrations being reached within 24 hour of exposure (Yue et al., 2016b). Comparable exposure levels, based on total silver, lead to significantly greater particle accumulation compared to silver ions, suggesting different routes of particle/ion uptake (Yue et al., 2016b). The role of exposure medium in silver nanoparticle/cell interactions and associated toxicity is another important consideration for quantifying particle accumulation and toxicity *in vitro* (Yue et al., 2015, Yue et al., 2016a).

With regard to intestinal cell/particle interactions, the only thus far available intestinal fish cell line, RTgutGC (Kawano et al., 2011), has proven very promising for particle uptake and translocation studies. It has been shown to form an epithelium with transepithelial electrical resistance (TEER) broadly similar to the gut epithelium *in vivo* (Sundell and Sundh, 2012). The cells also anatomically polarise as expected from the *in vivo* intestinal microenvironment, with (for example) apical expression of tight junction proteins, desmosomes and basolateral expression of the sodium-potassium ATPase (Geppert et al., 2016, Minghetti et al., 2017). Uptake and translocation of particles across the epithelium has been quantified in this system, including polystyrene NPs (Geppert et al., 2016), silver NPs (Geppert et al., 2020, Minghetti and Schirmer, 2016, Minghetti and Schirmer, 2019, Minghetti et al., 2019), titanium dioxide, copper and zinc oxide NPs (Geppert et al., 2020). Moreover, intracellular effects of silver NPs such as intracellular chemical transformations (Minghetti et al., 2019), inhibition of selenoenzymes (Chanda et al., 2021) and disruption of essential trace metal homeostasis (Minghetti and Schirmer, 2019) were measured in this system.

**Figure 8.1. Correlations between *ex vivo* exposure (mid intestine mucosa) and *in vivo* exposure (liver concentrations) with silver materials.**




Note: The data were ranked and then correlated. The $r^2$ values of the correlations were 0.9167, 0.8326 and 0.9142 for the $AgNO_3$, Ag NPs and $Ag_2S$ NPs treatments, respectively. The equation of the lines are (A) $y = 0.0073x + 92.812$, (B) $y = 0.0176x + 102.6s$ and (C) $y = 0.0013x + 8.6417$.

Source: The total Ag *in vivo* and *ex vivo* data are from Clark et al. (2019a) and Clark et al. (2019b).

The use of these cell lines as a true alternative to animals will require comparative analyses with systems that are closer to the *in vivo* situation, such as the gut sacs and OECD TG 305. Finally, based on their flexible application, cell lines may form the base for customized set-ups, such as flow-through on microfluidic chips (Drieschner et al., 2019a) that might enable some rapid screening of substances, and/or translocation through membranes that better mimic the base membrane in epithelia *in vivo* (Drieschner et al., 2019b, Drieschner et al., 2017). The RTgill-W1 cell line assay for predicting acute toxicity to fish is repeatable and reproducible (Fischer et al., 2019) and a standardised OECD method has been developed (Schirmer, 2020). The methodology is now available as OECD TG 249 (OECD, 2021). This illustrates that it is possible to adopt approaches with fish cells for regulatory use. It may be possible to adapt the RTgill-W1 cell line assay for bioaccumulation studies, as well as develop the existing gut cell lines from trout in a similar manner. Protocols for fish hepatocytes (liver cells) have also been optimised with the prediction of bioaccumulation potential of chemicals in mind (Fay et al., 2014), although not yet with MNs. However, ecotoxicity protocols using fish liver cells and MNs (Galbis-Martínez et al., 2018) might be adapted for a bioaccumulation endpoint. Clearly, fish cell culture has some considerable utility and could find applications in the screening of MNs for bioaccumulation potential.

# 9 *In vivo* dietary exposure in fish using TG 305 and the validity of *n*BMFs

In keeping with the 3Rs, the use of the *in vivo* test would be a last resort for MNs of concern. This section shows examples where TG 305 has been used to estimate a *n*BMF for a MN, and also considers how to interpret any *n*BMF obtained. As described earlier in this report, and reviewed in Handy et al. (2018), the TG 305 approach is being applied to MNs, especially using the existing dietary exposure method with minimal (e.g., additional metal salt controls) or no modification of the protocol for MNs. The purpose of the test is to measure the bioaccumulation potential *in vivo*. From the data sets analysed, it was possible to show that the total metal concentrations in the organs of fish from the relevant MN exposure was at or approaching an apparent equilibrium (Figure 5.1.). This enabled the calculation of *n*BMF estimates, along with other BMF values collected from the literature [Table 9.1, modified from (Handy et al., 2022)]. The *n*BMF values are often small (much less than 0.1) indicating that the MNs tested so far, are not very bioaccumulative, although a small rate of bioaccumulation could present a chronic hazard to fish, as is often the case for dissolved metals in the diet (Handy et al., 2005).

Data quality and reliability is also crucial as the *n*BMF values could be used in regulatory assessment. The Klimisch score for reliability (Klimisch et al., 1997) was applied to the available data sets used here and in (Handy et al., 2022). This very stringent approach to criteria for data mining was immediately problematic for collecting data from the published scientific literature, with very few studies meeting all the criteria (Handy et al., 2022); or the primary research aim of the published research paper was not about deriving a *n*BMF for regulatory use. For example, the dietary study of Ramsden et al. (2009) was not intended to calculate a *n*BMF for $TiO_2$, and with the background of Ti in commercial animal feed being impossible to remove without compromising the nutritional value of the feed, there was no strong evidence of the accumulation of ‘new’ Ti in the tissues.

## Table 9.1. Biomagnification factors (BMFs) from fish dietary exposures with metallic nanomaterials or metal salt controls.

| Material | Primary particle size | Nominal exposure concentration | Whole body or organ metal concentration (µg $g^{-1}$ dw) | BMF | Species | Comments | Authors |
|---|---|---|---|---|---|---|---|
| $AgNO_3$ | - | 100 mg $kg^{-1}$ | 3.925 ± 1.163* | 0.039 | *Oncorhynchus mykiss* | Treatment of interest was compared to an unexposed control. | (Clark et al., 2019a) |
| Ag NPs | 50 nm | 100 mg $kg^{-1}$ | 3.81 ± 0.796* | 0.038 | *Oncorhynchus mykiss* | Treatment of interest was compared to an unexposed control and dissolved metal control. | (Clark et al., 2019a) |
| $Ag_2S$ NPs | 20 nm | 100 mg $kg^{-1}$ | 0.321 ± 0.112* | 0.003 | *Oncorhynchus mykiss* | Treatment of interest was compared to an unexposed control and dissolved metal control. | (Clark et al., 2019a) |
| $CuSO_4$ | - | 750 mg $kg^{-1}$ | 2.941 ± 0.722* | 0.004 | *Oncorhynchus mykiss* | Treatment of interest was compared to an unexposed control | (Boyle et al., 2021) |
| CuO NPs | 18 nm | 750 mg $kg^{-1}$ | 2.849 ± 0.848* | 0.004 | *Oncorhynchus mykiss* | Treatment of interest was compared to an unexposed control and dissolved metal control. | (Boyle et al., 2021) |
| $TiO_2$ NPs | 21 nm | 10 or 100 mg $kg^{-1}$ | < 0.4 in major organs | Negligible for organs | *Oncorhynchus mykiss* | Biomagnification factor cannot be calculated as the organ concentrations for total Ti were very low or at background levels in most organs including skeletal muscle. No bulk material control in the study because of natural Ti background in the animal feed. | (Ramsden et al., 2009) |
| ZnO NPs | 25 nm | 50 or 500 mg $kg^{-1}$ | Background Zn 345- 9 µg $g^{-1}$ as wet weight in major organs | Negligible for organs | *Cyprinus carpio* | No difference in total Zn concentration compared to the control following 6 weeks dietary exposure to either 50 or 500 mg ZnO $kg^{-1}$. Thus, no appreciable Zn accumulation above the background. Skeletal muscle or carcass not reported so whole body concentration not possible to calculate. | (Chupani et al., 2018) |
| ZnO NPs | 20–30 nm | 300 or 1000 mg $kg^{-1}$ | ~ 40 µg $g^{-1}$ as fresh weight or less in the liver | 0.04 for the liver | *Oncorhynchus mykiss* | Only one time point showed slightly increased total Zn concentration in the liver of the treatments compared to the control following 11 days exposure to either 300 or 1000 mg ZnO $kg^{-1}$. Also, no dissolved metal controls. Skeletal muscle or carcass not reported so whole body concentration not possible to calculate. | (Connolly et al., 2016) |
| $TiO_2$ NPs | 21 nm | 0.1 mg $L^{-1}$ | 106.57 ± 14.89# | 0.024 | *Danio rerio* | Fed on a diet of contaminated *Daphnia* for 2 weeks. *Daphnia* were exposed to 0.1 mg $L^{-1}$ of $TiO_2$ for 24 h. | (Zhu et al., 2010) |
| $TiO_2$ NPs | 21 nm | 1.0 mg $L^{-1}$ | 522.02 ± 12.94# | 0.009 | *Danio rerio* | Fed on a diet of contaminated *Daphnia* for 2 weeks. *Daphnia* were exposed to 1.0 mg $L^{-1}$ of $TiO_2$ for 24 h. | (Zhu et al., 2010) |

Note: Data not applicable to the test material. * Whole body values calculated from the sum of the individual organs plus the remaining carcass. #Whole body values measured from acid digests of the whole fish, unclear if/how the gut was included in the measurement.
Source: Part of (Handy et al., 2022).

For some trace elements that are nutritionally required, the background concentration of metal in the tissues of 'unexposed' control fish in the laboratory is easily measured. This is especially relevant to Zn (Hogstrand and Wood, 1996) and Cu (Grosell et al., 1997). Consequently, exposures to ZnO NPs up to 1000 mg $kg^{-1}$ for 39 days did not result in a significantly elevated tissue zinc concentration (Chupani et al., 2018, Connolly et al., 2016); such that any BMF would be negligible or difficult to determine from the background. These problems are recognised, and many of the limitations identified for metals and other naturally occurring substances regarding the measurement of bioaccumulation factors may also apply to some MNs. Similar consideration for trace elements or background concentration needs to be applied to invertebrate bioaccumulation test where the nutritionally required metals (e.g., Zn, Cu, Fe) and minerals in hard structures (e.g., carapace) are readily detected.

One should also be clear on what any apparent *n*BMF has measured. In the majority of studies so far on fish, there is no measurement of particle number concentration in the tissue, except for the spICP-MS study on Ag MNs by Clark et al. (2019b). Therefore, it is not a *n*BMF for the nano form *per se*. Instead, it is a *n*BMFs calculated on the basis of total metal concentration in the tissue for a dietary exposure to a metal-containing MN. Notably, Clark et al. (2019c) found that exposure to $AgNO_3$ also resulted in the appearance of Ag-containing particles in the liver; perhaps due to AgCl particle formation in the gut lumen and/or biogenic particle formation in the tissue. If such processes are occurring in tissues, it may be difficult to determine a *n*BMF on the basis of particle number concentration, and this would rely on a dynamic steady-state between the exposure, biogenic particle formation and particle biodegradation. Currently, there are no internationally approved methods to extract and measure MNs in tissues for bioaccumulation measurements. However, effort is underway on the extraction methods for different MNs from tissues and their detection by single particle ICP-MS (Laycock et al., 2022) and with a document overviewing the possible approaches being prepared for the WPMN. The TG 305 also includes information on how to calculate a BCF or BMF using a kinetic method by essentially comparing the uptake and elimination rate constants. The equations (e.g., Annex 7 of TG 305 and elsewhere) rely on the mathematics for steady-state equilibria. It may be possible (in theory) to apply these equations to an apparent steady-state result based on total metal from a MN exposure, but it would be incorrect to use these equations for dynamic equilibria of particle number concentration in tissue without some modifications of the mathematics. Thus, if it is desirable to do kinetic calculations using particle number concentrations in the tissue, or similar particle metrics, then some modification and up-dating of the equations will likely be needed in TG 305.

It is important to consider that TG 305 was not written with MNs in mind, and so particle controls for any 'nano' effect are not specified in the protocol. Dissolved metal and/or bulk (micron scale) material controls have been used in dietary exposure studies on fish to inform on whether the bioaccumulation is related to particle size, or shape (Handy et al., 2022, Connolly et al., 2023). From the perspective of the 3Rs, the use of additional controls for MNs would use more fish, but this might be 'off-set' by applying the one concentration approach to TG 305 with MNs, although that would also need validating for MNs. The controls may not be needed for calculating a *n*BMF *per se*, and BMFs of metal salts for comparison may already be available in the scientific literature.

Other MN-specific concerns for the validity of TG 305 relate to sources of experimental variation in the study designs that are likely to alter particle behaviour and therefore uptake. For the waterborne exposure method, the precise details of the water chemistry (ionic strength, pH, divalent ion concentrations, natural organic matter) will influence the colloidal behaviour of the MNs (Handy et al., 2012) and so further guidance on allowable ranges of water chemistry variables for TG 305 may be needed for MNs. For dietary exposures to metals, it is well known

that temperature, ration size; as well as the stocking density of the fish and their ability to form social hierarchies for feeding, will alter the ingested dose of individual animals (Campbell et al., 2005). However, these factors have not been explored in any detail for MNs yet. A commercially available fish food (e.g., floating and/or slow sinking pelletized diet) that is characterised in terms of at least protein and fat content is recommended for dietary bioaccumulation studies according to TG 305. When spiking the food with the test substance, all possible efforts should be made to ensure homogeneity throughout the test diet, and this aspect is especially a concern for MNs that aggregate and may not be homogeneous. One common approach is to make a particle dispersion in ultrapure water and then to spray or mix it carefully with the dry food pellets, then top-coat the feed with a 10% gelatine solution to seal in the test material so that it does not leach into the aquarium water (Ramsden et al., 2009, Clark et al., 2019a). Individual pellets of the test diet should then be analysed to know if the food preparation was successful at dosing all of the food pellets, or at least sub-samples (~1-2 g) of the feed analysed to confirm the exposure concentration in the feed. However, specific spiking procedures for MNs need to be further developed and validated for standardisation in methods such as TG 305 (Connolly et al., 2023). In addition, some MNs can profoundly change the texture, buoyancy, or colour of the food making it less palatable to the fish [e.g., carbon nanotubes can make very hard pellets, (Fraser et al., 2011)]. So, more careful trials on feed acceptability to the fish may be needed for MNs in TG 305. Data on lipophilic MNs in fish feed are especially lacking.

# 10 Possible structure of a tiered testing strategy and a decision tree for moving between the tiers

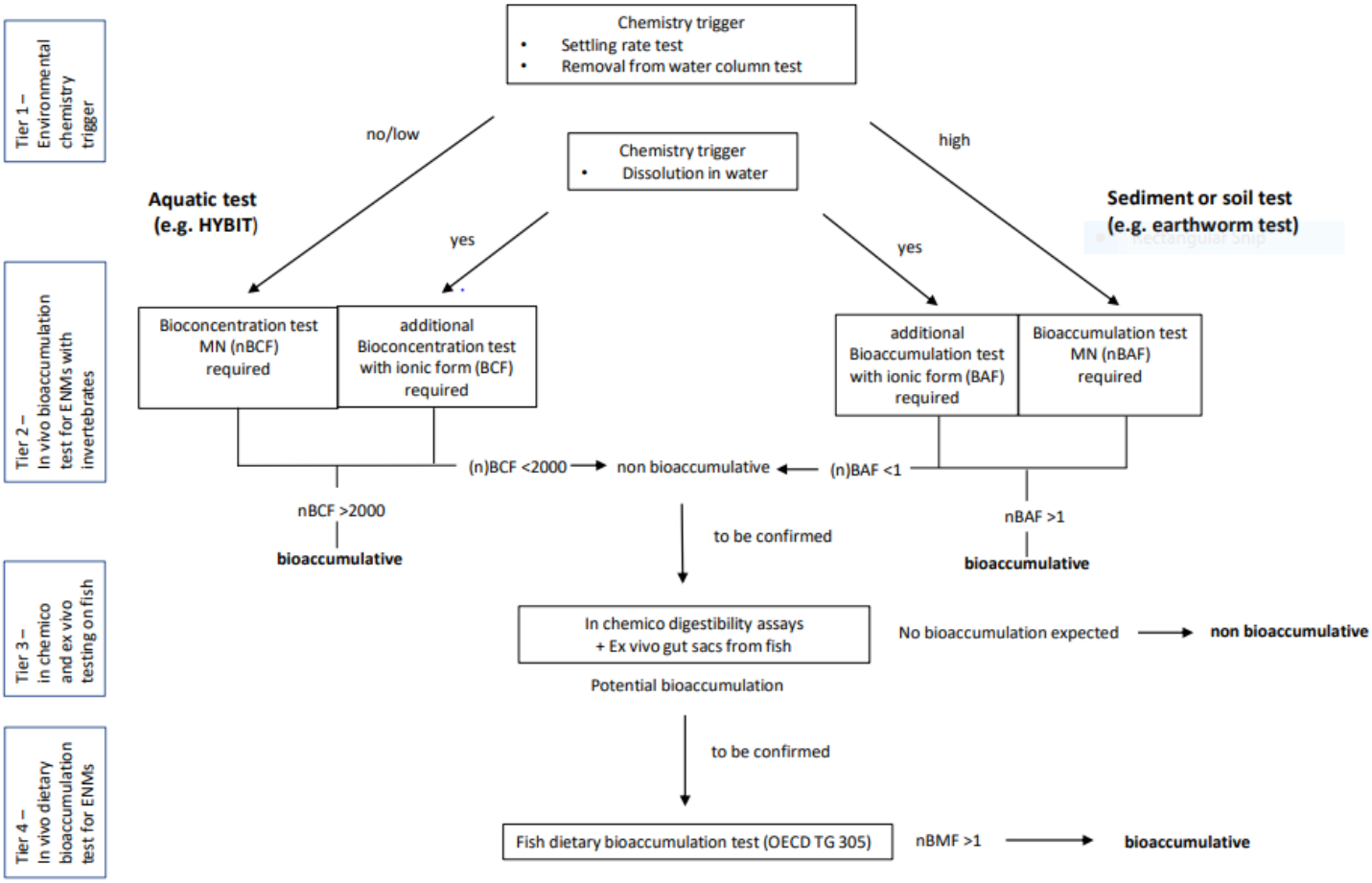
The notion of a tiered approach for determining the bioaccumulation potential of MNs in fish was first described by Handy et al. (2018), with aspects of navigation through such a scheme modified later (Handy et al., 2021), and with additional consideration of invertebrate testing, especially using *H. azteca*, suggested by Kuehr et al. (2021c). These schemes are shown in Annex A (Figures A.1 and A.2 respectively). The broad thinking was to have an initial tier which provided a chemical trigger that is relevant to the behaviour of MNs. In Tier 1, the chemical and physical properties of the MN are reviewed/investigated with a particular focus on dissolution or the release of ions from the MN into water or lipids, and the settling rate of the MN (Handy et al., 2021, Kuehr et al., 2021c). Tier 2 involves collecting existing data as evidence for a bioaccumulation concern from the literature, use of predictive computational tools, and with some non-vertebrate animals testing (i.e., invertebrates such as earthworms or freshwater amphipods). Tier 3 involves moving to '*in vitro*' alternatives to fish such as the digestibility assay and gut sacs, and perhaps fish cell culture studies for bioaccumulation, and a final tier 4 consisting of the dietary method of TG 305 for MNs (Handy et al., 2021, Handy et al., 2022). Importantly, these scientific papers show the overall broad concept and that there are a range of tools that could be used for a tiered approach to determining bioaccumulation potential, whilst minimising the use of vertebrate animals (i.e., fish).

An integrated approach to testing with tiers that enable the incorporation of the 3Rs, would need some kind of decision tree to guide the user through the scheme. One such approach, with different possibilities for specification of the tiers to illustrate the thinking is shown (Figure 10.1). It is generally agreed that the chemical trigger for starting the scheme should be a measurement that relates to the behaviour of MNs, which in turn, may inform on bioaccumulation potential. This could include aspects such as dissolution or settling rates from the dispersion (discussed earlier). If particle settling is positive (e.g., unstable dispersion where particles aggregate and settle from the water column) then one might choose a benthic (or terrestrial) test system instead of the aquatic test as the material may settle to contaminate the sediment and organisms living there. If part of the material dissolves in the water column, a bioaccumulation concern through exposure in the water column should be addressed by testing also the ionic form of the MN (Handy et al., 2021). If the MN completely dissolves, then one might exit the strategy and use the existing risk assessment for the dissolved substance. The result for the "chemistry trigger" (i.e., physical and chemical properties of the MNs) thus helps to select the right exposure pathway for further investigations on the next tier. This second tier could start by reviewing existing data sets on bioaccumulation and/or with some computational modelling to show a concern for bioaccumulation potential of the MN. The idea here is to explore existing data and to only move towards new experiments with animals when necessary. It is also

important to note that for some regulatory frameworks information on aquatic species are required.

### Figure 10.1. An example assessment scheme with a proposed decision tree for the bioaccumulation assessment for manufactured nanomaterials (MNs).

Expert judgment is required to come to a final decision and to employ the methods considered.




Note: Some of the suggested methods may need further standardisation and/or validation.

It would be very useful to include an invertebrate bioaccumulation test in tier 2 as an alternative to vertebrate animal testing. Here, this is illustrated with the TG 317 earthworm bioaccumulation test for terrestrial systems which seems to work well for MNs as well as the aquatic bioaccumulation test with *Hyallela azteca,* which has been developed recently (Kuehr et al., 2021b), or an oligochaete test for aquatic sediments such as TG 315. However, also further tests with other aquatic or terrestrial invertebrate species, might be available for such a scheme. The thinking is, if the invertebrate bioaccumulation tests is positive, then one might exit the scheme, with limited justification to move forwards to testing *in vivo* fish. If the invertebrate test(s) raised no concern or evidence of bioaccumulation ("non-B"), then one would move to the next tier in the scheme to confirm the result. However, defined threshold values are required to allow a clear assessment of the "non-B" or "B" scenario. In contrast to the earthworm bioaccumulation test (TG 317) and oligochaete bioaccumulation test (TG 315) which provides BAF values, the *Hyalella* test for instance allows to derive a BCF endpoint, which is fully regulatory applicable following the established threshold values [e.g., "B" equals BCF > 2000 in the EU Regulation REACH at least (ECHA, 2017b)]. Other thresholds or models for BCF to define a bioaccumulative substance in other parts of the world. Nonetheless, in principle, it is also possible to derive a BMF endpoint based on a modified *Hyalella* test (Kuehr et al. 2021).

In tier 3 (Handy et al., 2022), it is proposed that the *in chemico* digestibility assay for the fish gut lumen is carried out first (Annex A, Figure A.1, and in Figure 10.1). The endpoint of the assay is typically a percentage digestibility value, and for example a value of 5% might indicate that 1/20$^{th}$ of the ingested dose is bioaccessible or available for uptake. Again, some consensus building by the scientific community is needed to agree the threshold values for a positive outcome in this test, or if another similar *in chemico* assay should be used. For example, for dietary metals in fish, if the bioaccessible fractions are more than a few percent of the ingested dose, this may be a concern for bioaccumulation in the long term. Any threshold values would likely be specific for each (group of) substance(s), as they are for individual metals and other chemicals, and would need to be agreed by examining the data obtained. Whatever, the thinking is to show whether the MN would be bioaccessible and therefore a concern for oral uptake in a fish bioaccumulation test. In addition to the *in chemico* digestibility assay for fish gut lumen, still within tier 3 (the same tier), the potential for uptake would need to be further explored in *ex vivo* gut sacs of fish, using the mid or hind intestine, and preferably for the same species as would be used in an *in vivo* test according to TG 305. The gut sac assay, in theory, might enable the calculation of a *n*BMF for the intestine, or a similar metric on uptake to help with decision making on how to proceed through to the next tier, or not. The final tier in the scheme is TG 305, with suitable modifications for the MN of concern, as appropriate.

To harmonise with existing use of BMF values, existing regulatory guidance [for example, in the EU (ECHA, 2017a)] may be applied, but with some modifications for MNs. These modifications for MNs should be agreed with a *n*BMF > 1, allowing a clear assessment of the "B" scenario. On the other hand, with a *n*BMF < 1 obtained by means of the *ex vivo* gut sacs method the result of the preceding invertebrate test ("non-B") would be confirmed and no further tests required. However, if the test is positive, and thus in contradiction to the result of the preceding invertebrate test, then a fish study may still be required. Generally, with the availability of a carefully validated and regulatory accepted test strategy covering tiers 1-3, the need to carry out an *in vivo* fish test in tier 4 would be expected to be significantly reduced and it might even allow to end the use of fish for testing in the future. However, the *in chemico* digestibility assay and the *ex vivo* gut sacs method for testing MNs still need to be further standardised and to confirm their suitability for regulatory assessment of MNs. Especially, it needs to be proven that clearly defined endpoints allowing an unambiguous assessment of the "non-B" or "B" scenario can be derived. Following this, further validation approaches including inter-laboratory testing, are required to confirm the robustness and transferability of the methods.

# 11 Data gaps and aspects requiring further development for standardisation or application in nano safety testing

The aim of this section is to identify some topics that require further investigation and/or clarification in order to meet the requirements of regulatory testing around the world. The paragraphs below identify topics for further development.

*Chemistry trigger* – so far, there is no single particle metric that best predicts bioaccumulation potential in fish, but there are a few metrics, that together, give reasonably good predictions so far for metallic MNs. Agreement is needed on the computational methodology to best derive prediction equations and the certainty level needed in the predictions for regulatory use. Also, many of the physico-chemical data currently available are in the scientific literature, and not obtained using an approved OECD Test Guideline (TG). Some agreement is needed on the acceptability of physico-chemical data from the literature, and on how existing TGs on physico-chemical properties can be applied in this scheme. Notably, on the inclusion of the recent GD 318 on dissolution and dispersion stability of MNs (OECD, 2020b) in tier 1. Agreement about using the physico-chemical properties of MNs as a chemistry trigger(s) is also needed on the quantitative threshold (e.g., based on multiple regression or another appropriate descriptor) that should trigger the next tier. For example, if settling rate was used, what would be the appropriate settling rate to trigger the next tier, and so on.

*Invertebrate bioaccumulation tests* – such as the earthworm bioaccumulation test, OECD TG 317, and the sediment dwelling oligochaete test (OECD TG 315), as well as the recently developed bioaccumulation test with the freshwater amphipod *Hyalella azteca* have utility for MNs. However, other (aquatic) bioaccumulation tests may have potential to be further developed for regulatory use. The development and validation of bioaccumulation tests for MNs in marine species is also needed. A selection of additional relevant test methods is included in Annex B.

*In chemico digestibility assay and gut sacs* – both approaches have been shown here with a selection of metallic MNs, derived from essential and non-essential metals with different chemistries and mechanisms of uptake. This is a sufficient data set on ‘example materials’ to demonstrate the utility of the techniques for metallic MNs, but data sets are needed on carbon-based MNs including CNTs and nanoplastics. Both assays need to be developed for herbivorous fishes such as carp, as much of the current data is only for rainbow trout. Further standardisation and validation of the protocols would also enable efforts towards separate TGs, one for each approach.

*In vitro fish cell bioaccumulation tests* – Fish cell lines also offer an alternative to bioaccumulation testing *in vivo*, but the methodology for cell culture assays needs to be adapted to measure bioaccumulation, and this could include the development of assays with rainbow trout gill cells (‘waterborne’ exposures) and gut epithelial cells (‘dietary’ exposures). Fish liver cell assays could also be developed to assess

bioaccumulation potential. Accompanying standardised (OECD) guidance for these new tests would be needed to advise on appropriate quantitative triggers based on the endpoints presented in the tests to decide on the need to a conduct a fish bioaccumulation test for MNs. This guidance would also advise on the use and general applicability of these assays if more widely applied for general chemical safety assessment..

*Data quality in scientific publications* – the proposals here include using existing scientific data to help make a decision on whether to waive or proceed to TG 305. While the Klimisch score can be adopted, some further details on exactly how to score scientific papers for data quality on bioaccumulation for MNs is needed. This aspect of scientific paper quality is currently part of the discussions in the EU NanoHarmony project. There also needs to be some further considerations on thresholds for 'weight of evidence' i.e., exactly how much evidence is needed from the literature before a decision can be made to move to the next tier in the scheme. One possible interim solution could be to use very conservative thresholds (precaution) and re-evaluate the thresholds as more data or evidence becomes available.

*Computational approaches* – Technological advances including high content and high throughput screening and omics approaches have transformed nanosafety research into a data rich field. Nanoinformatics and machine learning-based *in silico* modelling is being applied to nanosafety, but this effort now also needs to be directed specifically at the issue of bioaccumulation potential. It is clear that computational approaches could be used for data gap filling and data interpolation on bioaccumulation, and this needs further exploration. However, for MN property models to be robust, predictive, and broadly applicable, large amounts of high-quality and complete experimental data are needed, that are organized and accessible (Afantitis et al., 2020). A current bottleneck is the fragmentation and inaccessibility of much of the data generated to date. To overcome this data fragmentation and facilitate model development, new processes need to be developed and implemented that will allow the capture of both the MN data and the associated metadata making the produced datasets findable, accessible, interoperable and re-usable (FAIR) (Papadiamantis et al., 2020b).

*Refinements to TG 305 and animal welfare* – the one concentration approach seems feasible for MNs, as it is for other chemicals. However, some dialogue is required to agree how this will be applied with any nano-specific caveats. For instance, in some cases it can be anticipated that the bioconcentration of a substance is dependent on the water concentration (e.g., for metals, where the uptake in fish may be at least partly regulated). In such a case it is necessary that at least two concentrations are tested (OECD TG 305). Further data sets to illustrate the utility of the one concentration approach for MNs are needed, and for different types of carbon-based MNs and nanoplastics.

*Measurement methods for organic nanomaterials in tissues* – this document has necessarily focussed on metal-containing MNs where the bioaccumulation could be inferred from the total metal concentrations in the tissues. While guidance for metal-containing MNs is possible, the lack of routine measurement methods for carbon-based MNs such as carbon nanotubes, fullerenes, organic micelles or liposomes, in tissues has hampered the study of bioaccumulation and remains a technical barrier to obtaining data for the calculation of *n*BMFs or *n*BCFs. In principle, any testing strategy should also work for organic forms of MNs, but effort is needed on the latter. Similarly, while spICP-MS has been used to identify metallic MNs in tissues, the extraction methods and detection protocols also need to be evolved for a wider range of MNs and also to standardise methodology for regulatory use.

# 12 Conclusions and recommendations

The meta-analysis and information presented here on metal-containing MNs shows that an integrated strategy with a tiered approach to bioaccumulation testing of MNs is feasible. Some possible examples of a tiered approach to bioaccumulation testing have been outlined here to illustrate the kind of thinking. However, the precise structure of any tiered system, how to navigate through it, and the thresholds used for that navigation would need to be agreed after selection, further development and/or validation of the appropriate tools and techniques mentioned herein. Nonetheless, it is generally agreed that chemical triggers in tier 1 that are more relevant to the behaviour of MNs should be used instead of the log $K_{ow}$ approach that does not work well for many MNs, and steady-state equilibria such as dissociation constants used for metals and other dissociating substances are not readily applicable to MNs. Chemical triggers based on particle size, hydrodynamic diameters of dispersions and/or settling behaviour, and dissolution are suggested to be used. Existing TGs and GDs could be considered in tier 1 of the scheme, notably the recent OECD GD 318 for the testing of dissolution and dispersion stability of MNs.

In tier 2 (i.e., one of the early tiers), it is generally agreed that bioaccumulation tests using invertebrates should be included in the strategy to help waive or include TG 305, so that only the MNs for which a concern has not been clarified are tested on fish. Bioaccumulation tests using aquatic invertebrates are being developed for MNs. Notably, the use of freshwater amphipod, *H. azteca*, is showing some utility. Furthermore, the bioaccumulation of MNs in the terrestrial system should be considered. The earthworm test, OECD TG 317, works well with MNs and therefore might have an immediate utility in a regulatory environment for MNs. The meta-analysis presented here also shows correlations between earthworm and fish data.

Regression analysis of data during meta-analysis can show simple predictions of lower tier data (e.g., physico-chemistry, earthworm data) with bioaccumulation in fish liver. However, it is desirable to develop and validate much more sophisticated computational tools for predicting the bioaccumulation of MNs in fish that use systems mathematics approaches, and data or text mining including artificial intelligence.

The *in chemico* digestibility assay has utility as a very rapid method to determine bioaccessible fractions, and along with uptake in gut sacs, could inform if an *in vivo* fish test is needed. The meta-analysis presented here showed regressions with correlations between gut sac results and *in vivo* fish liver for bioaccumulation in a selection of metallic MNs. Both the *in chemico* digestibility and gut sac assays would need some further standardisation and validation to make a TG for each method, if that was desirable to include in the overall strategy.

A possible scheme is described to illustrate the thinking around a tiered approach to testing and building on the ideas presented in the scientific literature. This includes consideration of the 3Rs, and options to exit the strategy when the evidence does not support moving to the *in vivo* bioaccumulation test. TG 305 should be a last resort. Crucially, it is intended that any agreed scheme should be precautionary and guard against false negatives and false positives.

## Recommendations

The following tasks are recommended to be performed within the suggested timescales:

### *Short term – to be implemented or completed within the next two to three years.*

- That a tiered approach to bioaccumulation testing is agreed and constructed with a detailed decision tree, following the principles set out in the example scheme(s) illustrated here, and so that there is more emphasis on the 3Rs and animal welfare, whilst remaining precautionary for chemical safety. This could take the form of a guidance document for the OECD.
- That the log $K_{ow}$ test is discontinued for MNs and instead, physico-chemical triggers based on particle size, hydrodynamic diameters of dispersions and/or settling behaviour, and dissolution are used instead. Some existing TGs and GDs could be considered in tier 1 of the scheme, notably the recent OECD GD 318 for the testing of dissolution and dispersion stability of MNs (OECD, 2020a) along with TG 318 on dispersion stability and the upcoming TG for dissolution in the aquatic environment Project 3.10 on the OECD Work Plan of the Test Guideline Programme.
- Simple multiple regression analysis of particle metrics should be explored with more data on other metallic MNs to provide even more reliable prediction equations for the likelihood of a bioaccumulation concern. Data on non-metallic MNs are also needed. The thresholds for prediction quality should be agreed, and how they are used in any decision tree. Furthermore, the thresholds for moving from one tier to the next should be agreed.
- The terrestrial earthworm bioaccumulation test, TG 317, and the aquatic *Hyalella azteca* bioaccumulation test work well for MNs, so far, and could be adopted into the scheme now, while other (aquatic) bioaccumulation tests are being developed. Validating the oligochaete sediment bioaccumulation test, TG 315, for MNs would also be helpful. Notably, effort should be spent on adapting and standardising at least one test using a marine invertebrate such as bivalves where the regulatory need specifies data requirements on a marine species.
- In keeping with the data requirements for environmental risk assessments in different regulations around the world, a ‘weight of evidence’ approach of a bioaccumulation concern should be adopted into the testing strategy for MNs. Data coming from existing OECD TGs, or other similar internationally validated protocols that have been shown to work for MNs, or designed for MNs, could be accepted in concordance with the Mutual Acceptance of Data (MAD) principle. Data from the scientific literature is a valuable resource that could be used in the context of providing a weight of evidence for decision making in a tiered approach to testing, as well as being supporting information, for environmental risk assessment. The Klimisch score can be adopted, with some modifications for MNs, to score scientific papers for data quality on bioaccumulation for MNs, so that such literature can be used reliably as part of the ‘weight of evidence’ approach in the testing scheme.
- When possible, the one concentration approach should be adopted in TG 305 to minimise the use of vertebrate animals in the test. Additional TG 305 tests for metal salt controls or bulk materials (traditional chemical forms) should only be included as an absolute last resort; when existing data are not available, or when bioaccumulation cannot be predicted from existing computational models. Every effort should be made to minimise the use of vertebrate animals in TG 305, and the use of animals generally in any tiered approach to testing.
- Further work is needed on the structure of the integrated and tiered approach to testing to firmly agree the details of each tier and how to navigate through them. Crucially, thresholds for ‘Yes’/’No’ decisions should be agreed with regard to moving to the next tier, and the precise

data conditions to exit the scheme within each tier should be agreed. The quantity and quality of data for a decision at each step in any decision tree should be agreed so that the scheme is robust. The scheme should remain conservative with respect to chemical safety (e.g., no false negatives), but minimise the workload and the use of animal testing.

- It would also be helpful to begin the processes of planning a validation exercise for any agreed scheme. This could include testing standardised (draft) protocols of each assay, and a mini round robin (inter-laboratory testing) of a few selected materials or methods.

### *Medium term – to be implemented or completed within the next 3-5 years.*

- While regression analysis and other straightforward and pragmatic meta-analysis may help in the immediate and medium term to provide predictions to aid decision making, effort should also focus on developing more sophisticated *in silico* computational tools for predicting the bioaccumulation of MNs; such as those evolving in the NanoSolveIT project. The use of PBPK modelling and read-across approaches for species of organism including mammals and invertebrates, and to group the MNs by critical physico-chemical properties should be considered.
- A fish digestibility assay should be further standardised and validated as a rapid tool for estimating bioaccessible fractions of MNs, and other chemicals. Similarly, a simplified protocol of the gut sac technique could also be standardised with regulatory use in mind.
- Bioaccumulation studies on MNs with the RTgill-W1 and the RTgutGC cell lines to simulate waterborne and dietary exposures respectively should be further explored (for example, based on or by adapting OECD TG 249 on fish cell line acute toxicity for bioaccumulation).

### *Long term – to be implemented or completed within the next decade.*

- It is expected that any scheme would be periodically reviewed and refined as data sets emerge, to both improve and simplify the scheme.
- A meta-analysis of the data and use of the scheme should be conducted to determine whether there is enough scientific evidence and support from the scientific community to withdraw assays that use vertebrate animals in the long term, including TG 305.
- Explore extending the scheme to other chemicals, should it be proven to work well for MNs.

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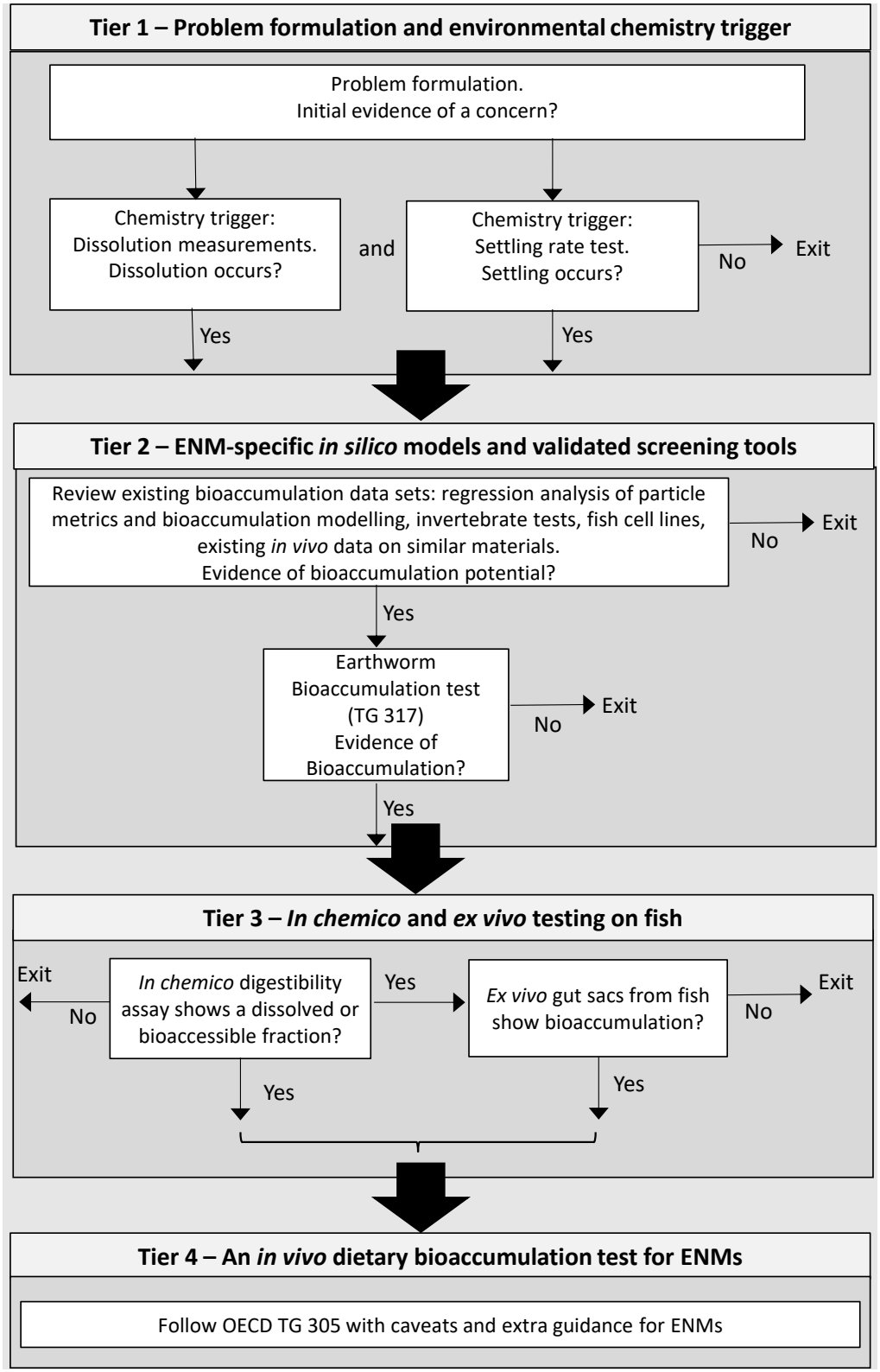
# Annex A. Example tiered approaches to bioaccumulation testing from the scientific literature.

**Figure A A.1. A proposed decision tree for working through the bioaccumulation testing strategy for manufactured nanomaterials (MNs), with scientific exit points in the early tiers.**

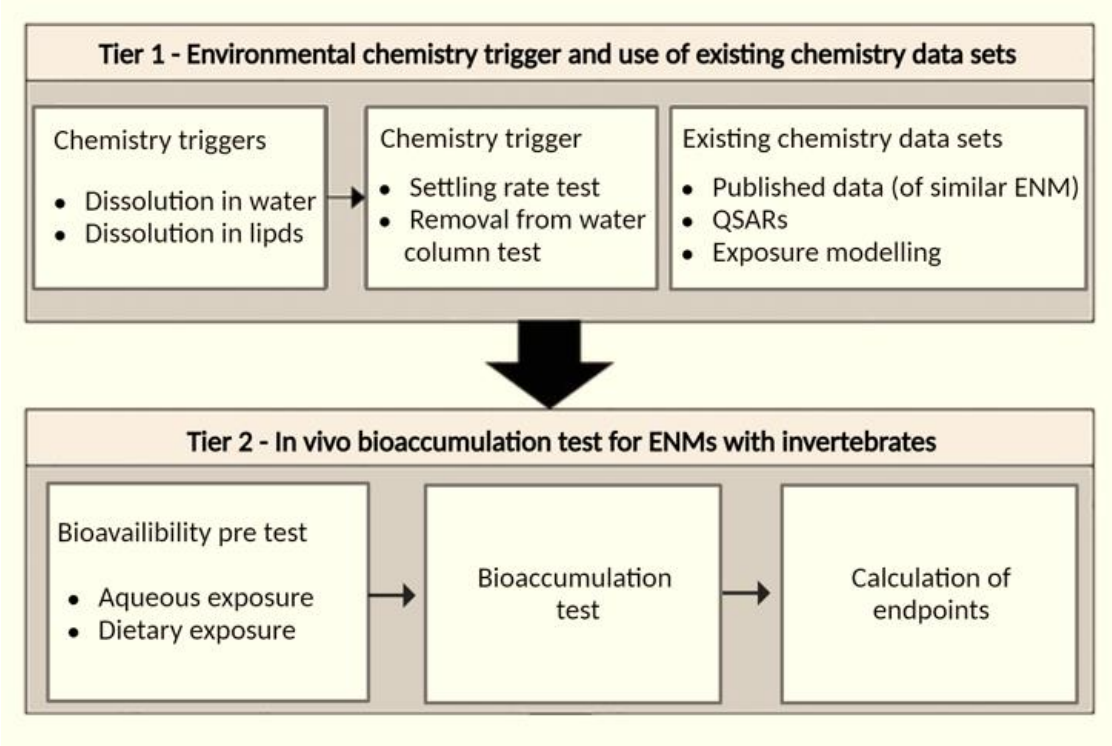



Note: ENMs; "Engineered Nanomaterials."
Source: As explained by Handy et al. (2021) and derived from the original scheme proposed by (Handy et al., 2018).

A tiered approach to bioaccumulation testing for MNs was first proposed in the scientific literature by (Handy et al., 2018) to show the overall concept and on how such a scheme could be possible. This

was revised in 2021 (Figure A.1) to draw particular attention to the utility of earthworm bioaccumulation tests for MNs and to begin to consider in more detail how to move between the tiers in such a scheme (Handy et al., 2021). Further data to illustrate the utility of the tools in tier 3 (digestibility assays, gut sacs of fish) were described in a meta-analysis (Handy et al., 2022) and to consider how the higher tiers in such a scheme could work.

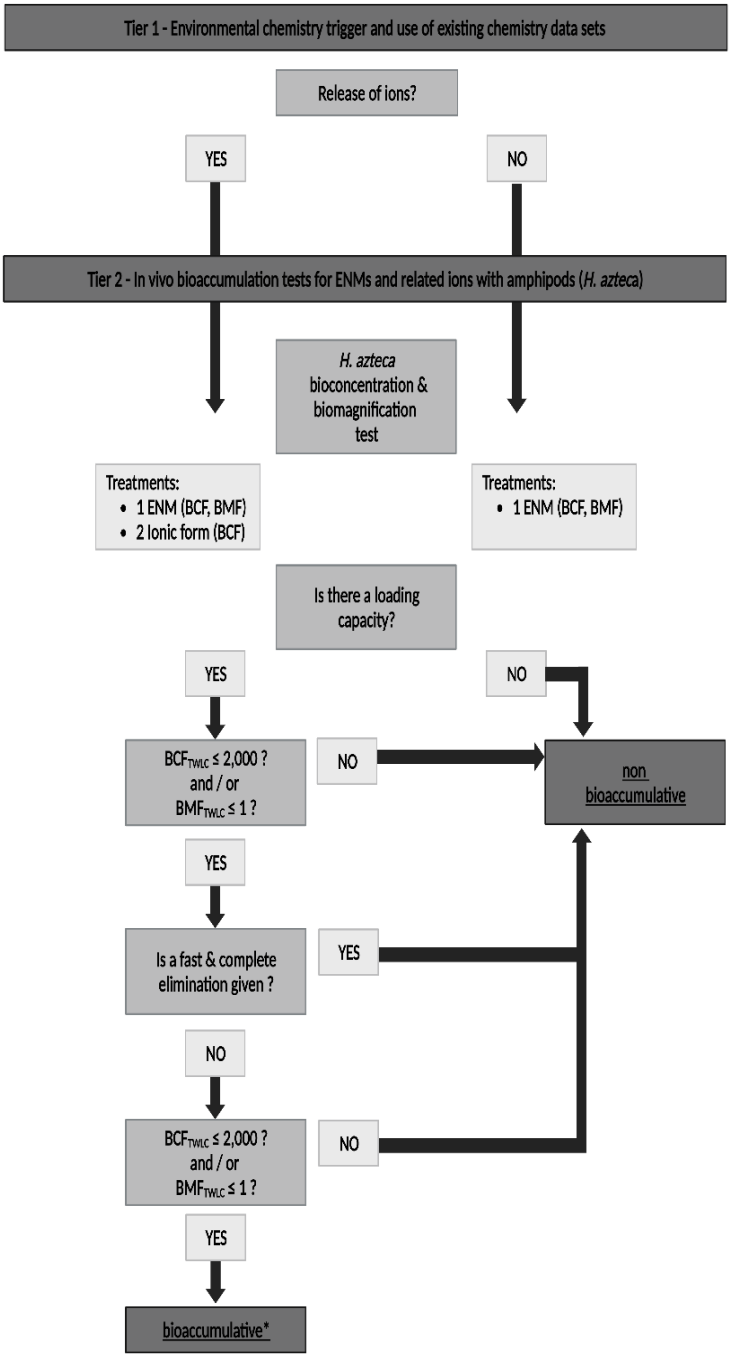
**Figure A A.2. A modification of the scheme proposed by Handy et al. (2018) to include tests for invertebrates, and especially considering inclusion of bioaccumulation tests using *H. azteca.***




Source: Figure modified from (Kuehr et al., 2021c).

The role of aquatic invertebrate bioaccumulation tests for MNs in a tiered approach to testing have been given some special attention in the scientific literature (Kuehr et al., 2021c) with a modified proposal of the tier 2 (Figure A.2). As a first step (pre-test) of the second tier for invertebrates, the bioavailability of the test compound to *H. azteca* needs to be estimated. Exposure concentrations should be selected which do not produce toxic effects at the dose level used, but still result in measurable tissue concentrations. *H. azteca* have no protection mechanisms to avoid exposure to the surrounding medium as observed for bivalves (e.g., valve closing and decreased filtration activity) and the determination of the exposure dose is thus comparatively simple. Details of a potential bioaccumulation test with *H. azteca* are described in a related scientific paper (Kuehr et al., 2021b) and a TG for this test is anticipated to be published soon (TGP Project 3.19).

## Figure A A.3. Assessment scheme for bioaccumulation assessment of MNs with amphipods (*H. azteca*)




Note: *if $BCF_{TWRC}$ is ≥ 2000 or ≥ 5000, and $BMF_{TWRC}$ ≥ 1, the test item is classified as 'bioaccumulative' or 'very bioaccumulative,' respectively, and a higher tier test could follow to refine the calculated endpoint. Bioconcentration (BCF) and biomagnification (BMF) factors are calculated based on the time-weighted residual capacity (TWRC) and the exposure concentrations. ENM; engineered nanomaterial. TWLC; time-weighted loading capacity.
Source: See (Kuehr et al., 2021c) for more details.

It is also important to including an assessment scheme with the aim of defining a 'bioaccumulative' or 'non-bioaccumulative' grading for MNs without using fish (Figure A.3). The suggested approach is not in competition with other concepts (e.g., Figure A.1), but provides additional possibilities which might be considered for the development of a 'unified' approach to a tiered testing scheme, including all possibilities (earthworms, *H. azteca*, and maybe others) so that the scheme has wide applicability to MNs for terrestrial, freshwater and marine applications. A decision tree can provide advice under which conditions or circumstances one should select a certain test(s) to move through the scheme.

Using the scheme outlined in Figure A.3, after following the exposure for 7 days as part of the pre-test, the body burden of the animals is measured to find out whether a significant loading capacity for the tested MN is observed. If so, then further bioconcentration and biomagnification studies (main tests) for the MN and, if necessary, a bioconcentration test for the ionic/dissolved form should be carried out. These tests must include a depuration phase to allow estimation of the half-life of the previously ingested/accumulated MNs, and to account for any rapid 'depuration' of the gut lumen contents which is not part of the true net uptake by the tissues. The uptake phase of the bioaccumulation tests (aqueous and/or dietary exposures) should last at least as long as required that three subsequent body burden measurements ($\Delta t \geq 12$ h) show no variation higher than 20% allowing the calculation of a time-weighted loading capacity (TWLC) for the end of the uptake phase based on the three last measurements. As a result of the main study, bioconcentration and biomagnification factors are then calculated by dividing TWLC by the exposure concentration in water or food, respectively. If the values are below the threshold value of 2000 ($BCF_{TWLC}$) or 1 ($BMF_{TWLC}$), the tested MN can be graded as 'non-bioaccumulative,' provided that these common threshold values are accepted for MN bioaccumulation assessment.

Generally, if one of the calculated endpoints shows a value above the set thresholds at the end of the uptake phase (main test), the results need to be further validated based on the depuration behaviour of the previously accumulated test item. Thus, the body burden of the animals needs to be further elucidated during a depuration phase following exposure. If the elimination of the previously measured MNs occurs fast and completely (half-life of < 1 day), the body burden and the loading capacity may primarily result from simply ingested, but not incorporated, and thus not bioaccumulated material, i.e., located in the gut (content) or attached to the animals' surface. This outcome would lead to a decision for a 'non-bioaccumulative' grading. If the elimination half-life is > 1 day, the depuration phase is extended until, either the burden reaches the initial natural background concentration, or three subsequent body burden measurements ($\Delta t \geq 12$ h) show that the residual body burden values do not vary by more than 20% (sink). Under these conditions, bioconcentration and biomagnification factors are calculated based on the time-weighted residual capacity (TWRC) (i.e., the last three measurements in the depuration phase) and the previously applied exposure concentrations. Again, the MN is graded as 'bioaccumulative' if these values are above 2000 for $BCF_{TWRC}$ and 1 for $BMF_{TWRC}$. If only the ionic/dissolved form shows $BCF_{TWLC/TWRC}$ values above the threshold of 2000, but not the respective MN tested in the bioconcentration approach, the MN has to be considered as 'non-bioaccumulative.' Due to the special properties of MN, the classical endpoints gained in bioaccumulation studies with MNs, like BCF or BMF cannot or should not be derived as is normally the case for water-soluble/non particulate test items. However, the proposed modified endpoints calculated based on TWLC and TWRC estimates should be more robust regarding the analytical challenges involved in MN testing. In addition to that, determining bioaccumulation of MNs under consideration of loading capacity and residuals will take into account and acknowledge specific, potentially ecological relevant behaviour of MNs within the assessment.

# Annex B. A selection of relevant test methods for bioaccumulation testing in fish and alternatives.

The following selection of test methods are intended to indicate possible alternative approaches to *in vivo* fish studies, for example using invertebrates or fish tissue/cell lines. It is not an exhaustive list from the scientific literature, but exemplar of the most relevant methods for bioaccumulation in an ecological context. Mammalian bioaccumulation tests are not included.

| Test method | Test species | Description | Development status | Test endpoint | Notes |
|---|---|---|---|---|---|
| | | | ***In vivo* testing - Aquatic and sediment species** | | |
| OECD TG305[1] | Fish such as zebra fish (*Danio rerio*), common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and others. | Aqueous and dietary exposures with uptake and depuration stages. | Published guideline. | Bioconcentration factor (BCF) or biomagnification factor (BMF) | Widely used flow-through study, including with nanomaterials. |
| *Hyalella azteca* bioconcentration test (HYBIT)[2,3,4] | Freshwater amphipod (*Hyalella azteca*). | Aqueous and dietary exposures with uptake and depuration stages. | Guideline under revision | BCF and BMF | Test used with organic chemicals, metal and metal oxide nanomaterials. |

| Test method | Test species | Description | Development status | Test endpoint | Notes |
|---|---|---|---|---|---|
| *ASTM E1022-22[5] | Marine bivalves such as the blue Mussel (*Mytilus edulis*), the scallop (*Pecten* spp.) and the oyster (*Crassostrea gigas* or *C. virginica*). | Aqueous exposure with uptake and depuration stages. | Published guideline. | BCF | Applicable to chemicals substances that can be measured accurately in water. |
| *Corbicula fluminea* flow-through test[6] | Freshwater filter-feeding bivalve (*Corbicula fluminea*) | Flow-through system that allows a continuous and constant exposure of test substances. | Test method in development. | BCF | Test method shows feasibility for testing with Ag and $TiO_2$ nanoforms in filtering organisms. |
| ***In vivo testing - Terrestrial animal species and plants*** | | | | | |
| OCSPP 850.4800[7] | Two plant species of potentially differing sensitivity, such as a monocotyledonous and a dicotyledonous species. | Uptake and translocation, of test substances and mixtures in terrestrial plants. | Published guideline. | Concentrations of free parent test substance, metabolites and soluble residues, and bound residues in pooled plant organs and pooled whole plants. | Water soluble test substances. Water insoluble test substances should be dissolved in an appropriate solvent. |

| Test method | Test species | Description | Development status | Test endpoint | Notes |
|---|---|---|---|---|---|
| OECD TG315[8] | Sediment-dwelling benthic oligochaetes such as *Lumbriculus variegatus* an inhabitant of freshwater sediments. | Uptake (exposure) phase and elimination (post-exposure) phase. | Published guideline. | Bioaccumulation factor (BAF) | Test applicable to organic chemicals and sediment-associated, stable metallo-organic compound. Metals can be measured with modifications to the test design. Adapted to work with nanomaterials. |
| OECD TG317[9] | Soil-ingesting terrestrial oligochaetes such as *Eisenia fetida* or *Eisenia andrei* (Lumbricidae). | Uptake (exposure) phase and elimination (post-exposure) phase. | Published guideline. | BAF | Test applicable to organic chemicals and soil-associated, stable metallo-organic compound. Metals can be measured with modifications to the test design. Adapted to work with nanomaterials. |
| | | | ***In vitro* testing** | | |
| OECD TG319A[10] | Rainbow trout (*Oncorhynchus mykiss*) liver tissue | Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes (RT-HEP) | Published guideline. | *In vitro* intrinsic clearance can be used to inform *in silico* prediction models of the bioaccumulation of substances in fish. | |

| Test method | Test species | Description | Development status | Test endpoint | Notes |
|---|---|---|---|---|---|
| OECD TG319B[11] | | Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout liver S9 sub-cellular fraction (RT-S9) | Published guideline. | *In vitro* intrinsic clearance can be used to inform *in silico* prediction models of the bioaccumulation of substances in fish. | |
| OECD TG249[12] | Cell line from rainbow trout (*Oncorhynchus mykiss*) gill, RTgill-W1 | Fish Cell Line Acute Toxicity: The RTgill-W1 cell line assay | Published guideline. | Test method predicts fish acute toxicity in product testing, it can be used for range-finding and pre-screening prior to fish-based toxicity tests, and the derived data can be used for hazard assessment in combination with other lines of evidence. | |
| ***In chemico* testing** | | | | | |
| *In chemico* digestibility assay[13] | Not applicable. | Simulates the digestive processes of the fish gut. A step-wise extraction of potentially bioavailable fractions in the gastrointestinal tract. | Test method in development. | Test method can determine the bioavailable fraction of dissolved substances. Method works with metal-based nanomaterials. | |

| Test method | Test species | Description | Development status | Test endpoint | Notes |
|---|---|---|---|---|---|
| | | | ***Ex vivo* testing** | | |
| Gut sac method[14] | Rainbow trout (*Oncorhynchus mykiss*) whole gut | The lumen within the fish gut is filled with the test substance of interest. | Test method in development. | | A close match to the gut barrier as it would exist *in vivo* and can precisely identify which regions of the gut are of concern. Method works with metal-based nanomaterials. |

** Note, ASTM 2022 replaced the 2013 protocol version in June 2022.*

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