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Foreword

This new guidance document is the outcome of a project jointly led by France, Spain and the United Kingdom. The scope of the project was to develop guidance on the study of bioaccumulation of manufactured nanomaterials in fish via water and via the diet. The focus of the guidance is the adequate conduct of TG 305 and the use of data for BMF and BCF determination, but not the waiving of the test. The document mostly concerns testing of metallic nanomaterials and does not address non-metallic nanomaterials given the difficulties in their determination.

Spain, with support from France and the United Kingdom developed this short guidance. Spain presented a first draft of the guidance document in June 2024 and the Joint WNT-WPMN Expert Group reviewed the document, and discussions were organised via virtual meetings to consolidate the draft guidance Document. The WNT reviewed the document in 2024 and approved it in April 2025 at its annual meeting.

Following declassification in July 2025, the guidance document is published under the responsibility of the Chemicals and Biotechnology Committee.

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Background

At an OECD expert meeting on ecotoxicology and environmental fate testing (OECD, 2014), the applicability of the well-established OECD Test Guideline 305 (TG 305) for the testing of bioaccumulation of nanomaterials in fish was discussed. It was agreed that the octanol-water partition coefficient test is problematic for many nanomaterials and that an alternative trigger to waive bioaccumulation testing was needed. Nanomaterials cannot reach thermodynamic equilibrium by distributing between two phases, water and n-octanol, due to their particulate nature. This renders the use of the octanol-water partition coefficient unsuitable for predicting the fate of nanomaterials (Praetorius et al 2014, Cornelis, 2015). Therefore, the regulatory use of the octanol-water partition coefficient is generally discouraged for nanomaterials (Handy et al. 2018, ECHA 2022) and render this coefficient unsuitable as a waiver for TG 305.

To address these two issues, i.e., how to waive testing in fish and how to test bioaccumulation of nanomaterials in fish, the United Kingdom, Spain and Finland proposed a project for the development of a "New Guidance Document (GD) on Assessing the Apparent Accumulation Potential of Nanomaterials". The issues with the octanol-water partition coefficient render this coefficient unsuitable as a waiver for TG 305. In search of other waivers, a tiered approach is being considered in parallel by the United Kingdom. As a first step in this process, a scoping review has been published in the Series on the Safety of Manufactured Nanomaterials No. 110 (OECD, 2024). This document described a tiered approach with four tiers. Briefly in Tier 1, the chemical and physical properties of the nanomaterial are reviewed/investigated with a particular focus on dissolution or the release of ions from the nanomaterial into water or lipids, and the settling rate of the nanomaterial. 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 nanomaterials".

Since 2018, the work focused on the applicability of TG 305 to nanomaterials is led by Spain. The experimental work has been supported by the H2020 project Gov4Nano (Europe's Horizon 2020 Research and Innovation Programme, Grant Agreement No. 814401) and Spanish resources. While the final tier of the scoping review directs only to dietary route for bioaccumulation test in fish for nanomaterials, the current document provides guidance on using TG 305 for nanomaterials more general, including whether the aqueous exposure may be applicable and when dietary methods are preferred. This guidance document (GD) only becomes relevant once it has been decided that a fish test is needed. The GD will provide guidance for all nanomaterials, but current technical limitations for the determination of non-metal-based nanomaterials in feed and fish tissues hamper detailed guidance in some sections for these nanomaterials compared to metal-based nanomaterials.

This document was led by Spain with contributions from the Netherlands and the United Kingdom and benefitted from the inputs of the Joint WNT/WPMN Expert Group on Ecotoxicity and Environmental Fate Testing. This Guidance Document could be subjected to amendments and refinements as new techniques

to measure non-metal-based nanomaterials emerge and further data on bioaccumulation of nanomaterials are available.

For a glossary of terms, please, refer to the glossary appearing in TG 305.

Introduction

1. The applicability of OECD Test Guideline 305 (TG 305) (OECD 2012) to conduct bioaccumulation assays in fish with nanomaterials needs additional guidance to address issues identified at an expert meeting on ecotoxicity and environmental fate testing (OECD 2014) and discussed in detail in Handy et al. (2018). In addition, some gaps in the performance of the studies of bioaccumulation in fish conducted with nanomaterials via water and via diet were identified in Connolly et al. (2023). These gaps hampered overall conclusions about the bioaccumulation potential of these forms of substances. For this reason, harmonisation on the design of the bioaccumulation potential measurement *in vivo* is needed.

2. Nanomaterials in aqueous media are in suspension and particle settling in the media provides a challenge for maintaining stable exposure conditions. Consequently, it is essential to determine the dispersion stability of the nanomaterials when an aqueous exposure approach is used. When the dispersion is not sufficiently stable, the dietary route should be applied as indicated in TG 305 (OECD 2012) for substances with very low water solubility. Taking dispersion stability into account, Section 3.1 of this document provides guidance on when the dietary exposure is recommended. The feasibility of the spiking of fish feed with nanomaterials also needs some considerations (see Chapter 5).

3. In addition, it is important to carefully consider the most appropriate and relevant exposure route as kinetic differences have been identified for metal oxide nanomaterials when administered via water or feed (Connolly et al. 2023; Johnston et al. 2010, Kalman et al. 2023, 2025; Zhu et al. 2010). This phenomenon is well known for dissolved metals that show faster uptake at the gills compared to the gut (Kamunde et al. 2002). The selection of the exposure route for nanomaterials should be based on the expected environmental relevance of the aqueous versus dietary exposure, and the practical considerations of maintaining the exposure levels for a valid test.

4. In terms of determination of the concentration of the nanomaterial in water, feed and fish, there are some important technical challenges to overcome. Guidance for the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies is forthcoming to address these challenges (the GD is expected to be published in 2026). For metal and metal oxide nanomaterials, the total metal concentration in the tissues, feed, or water, can be measured by traditional flame atomic absorption spectroscopy, or more commonly by inductively-coupled plasma optical emission spectroscopy (ICP-OES, e.g., TiO_2, Shaw et al. 2013), and/or mass spectrometry (ICP-MS, Clark et al. 2019). However, the identification of the form of the nanomaterial (presence of metal ions, single nanoparticles or agglomerates) requires further considerations. For example, the use of single particle ICP-MS (spICP-MS) allows for the detection of both dissolved and particulate fractions in tissue (Clark et al. 2021; Laycock et al. 2022), although this technique is currently mainly used at research level. Since additional guidance on how to use spICP-MS for nanomaterials will be provided in the GD mentioned before (expected in 2026) and an ISO Technical Specification is available (ISO 2024), its use could change in the future. For non-metal based nanomaterials, in particular for carbon-based nanomaterials, the approaches to measure concentrations in feed or tissues are limited, which leads to limitations in the application of TG 305 (OECD 2012) for this kind of nanomaterials. The use of radiolabelling (Petersen et al. 2023) is a possibility for the quantification

of carbon-based nanomaterials, but this is currently extremely expensive and implies important restrictions in relation to the use of special facilities.

5. The bioaccumulation kinetics and approaches provided in TG 305 (OECD 2012) to derive bioconcentration and biomagnification factors (BCF and BMF, respectively) were originally intended for solutes, and may not be appropriate for nanomaterials. The underlying assumptions of steady-state equilibria may not apply to any nanomaterial, which also may have different diffusion rates and membrane transport mechanisms compared to those of soluble chemicals (Handy et al. 2018).

6. Bioaccumulation data are required for the purposes of classification (according to the Globally Harmonised System), and risk and PBT (persistent, bioaccumulative, toxic) assessment. In these cases, some thresholds are derived from numerical values of BCF. These can be calculated directly from an aqueous exposure test, but not directly from a dietary test. Such BCFs must be derived from the observed dietary BMFs. For that, three main approaches are proposed in Annex 8 of TG 305 (OECD 2012) and in its accompanying Guidance Document No. 264 (GD 264) (OECD 2017a). Where these methods rely on the octanol-water partition coefficient they are not applicable for nanomaterials.

SCOPE

7. The current document is aimed to provide specific advice on how to perform bioaccumulation tests in fish with nanomaterials following TG 305 (OECD 2012), once it has been decided that such a fish test is needed. The purpose is to offer guidance on the critical points indicated in the Introduction section to generate harmonised results which can inform reliably on the accumulation potential of nanomaterials.

8. Considerations about the most appropriate testing regime (i.e. aqueous *versus* dietary), guidance for the preparation of stable and reproducible suspensions, indications on the appropriateness of the approaches proposed to derive BCF and BMF, the validity of BMF to assess the bioaccumulation of nanomaterials and the availability of methods to determine tissue/fish concentrations will be presented in the document.

Specific limitations on scope

9. The present GD is based on general accepted principles to assess the bioaccumulation of tested chemicals in fish. However, manufactured nanomaterials have particular physico-chemical properties that need specific approaches in the testing process. As many of these manufactured nanomaterials may retain their nanospecific properties, e.g. particulate nature, featuring low to no solubility, a different approach than that used for soluble chemicals is required. The plethora of manufactured nanomaterials results in specific cases that may require a bespoke approach to ensure an appropriate bioaccumulation testing. Therefore, this GD provides considerations to the user in order to facilitate the testing process.

10. It must be emphasized that currently there are technical limitations for an accurate quantification of carbon-based materials in organic matrices, including (fish) tissues and (fish) feed. This imposes also challenges for the assessment of the bioaccumulation of this kind of materials.

11. Finally, it must be taken into account that the continuous scientific and technological progress leads to the appearance of new materials (e.g. the so-called advanced materials) with specific properties that may also require specific considerations. Although such advanced materials can encompass a wide range of different types (see e.g. OECD 2022a, Giese et al. 2020), for many of these materials considerations will include challenges in exposing the fish (either via aqueous or dietary route) and quantification of the materials in the relevant media. Guidance in this document for nanomaterials may often be applicable, but specific properties of the (advanced) test material should be considered.

12. Considering all of the above it is recommended to ensure knowledge on the state of the art on analytical techniques (e.g. by performing a literature review), to consider published standard methods (e.g. those published by ISO, OECD TG 125, etc.) and to consult with analytical experts on the latest methods. In the near future, also the "GD on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies" will provide valuable guidance to be consulted. In some cases, specific situations, and geographical regions, consultation with regulatory authorities (if possible) may be desirable to determine data acceptability requirements prior to expending resources on testing.

CONSIDERATIONS FOR BIOACCUMULATION ASSAY DESIGN

Selection of the exposure route set-up

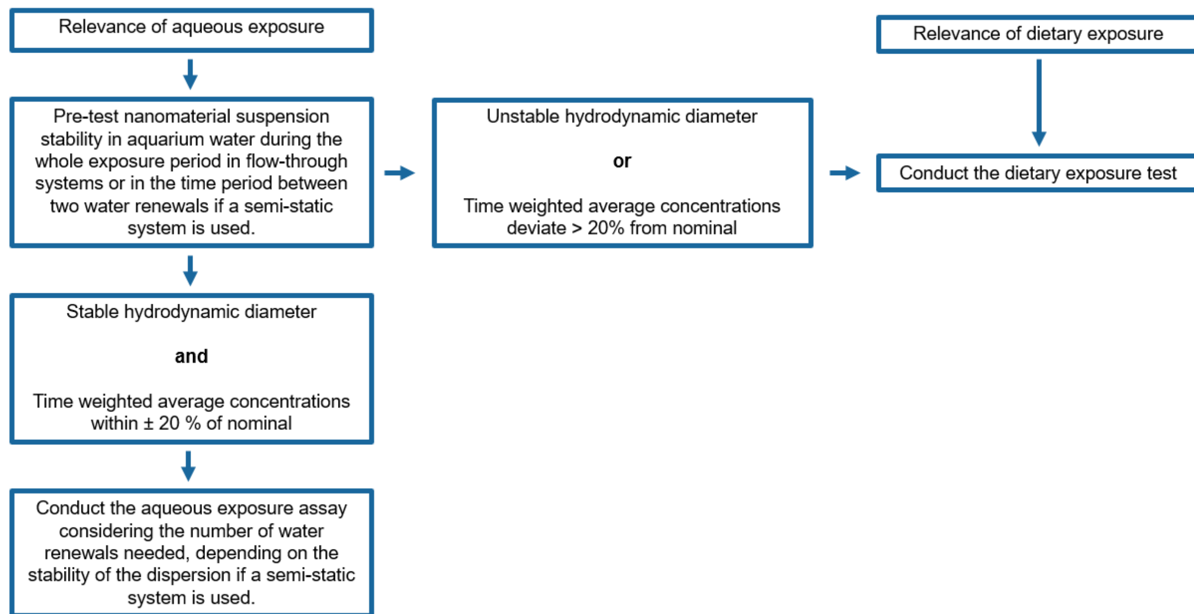
13. In 2012, TG 305 (OECD 2012) incorporated a dietary bioaccumulation test suitable for determining the bioaccumulation potential of substances with very low water solubility. The selection of the testing regime should be based on the solubility and stability of the test substance in water. As stated in TG 305 (paragraph 10), for substances where the solubility or the maintenance of the aqueous concentration as well as the analysis of these concentrations do not pose any constraints to the realization of an aqueous exposure method, this method is preferred to determine the bioconcentration potential of the substance. If a stable concentration of the test substance in water cannot be demonstrated, an aqueous study would not be appropriate, and the dietary approach for testing the substance in fish would be required (although interpretation and use of the results of the dietary test may depend on the regulatory framework) (Paragraph 5, TG 305) (OECD 2012).

14. Nanomaterials are often not readily soluble in water but can be dispersed stably in water. OECD GD 318 (OECD 2021) indicates in section 6.7, paragraph 138, that the information from dissolution, dispersion stability, and heteroagglomeration could be used for decision making prior to testing bioaccumulation. In addition, it has been shown that the kinetics of the nanomaterials can be different depending on the route of exposure used (Connolly et al. 2023; Johnston et al. 2010, Kalman et al. 2023, 2025; Zhu et al. 2010). The selection of the exposure route for nanomaterials in a bioaccumulation test should be based on the stability of the nanomaterial dispersed in the aquarium water and on the expected environmental relevance of the aqueous versus dietary exposure. For instance, the bioaccumulation in fish of nanomaterials used in fish feed formulations should be studied following a dietary exposure. Nanomaterials for which it is known that they accumulate in sediments, a dietary exposure in fish is recommended to study their bioaccumulation.

15. Independent of the chosen exposure route, TG 305 (OECD 2012) recommends the use of flow-through systems and semi-static regimes are permissible, provided that the validity criteria (*cf.* paragraphs 24 and 113 in TG 305) are satisfied. In the dietary exposure route, the flow-through system is not necessary to maintain aqueous concentrations of the tested substance, but it will help maintain adequate dissolved oxygen concentrations and help ensure clean water and remove influences of *e.g.* excretion products. However, as indicated in GD 317 (OECD 2022b), when a flow-through system is used, caution should be applied as nanomaterials may be lost to tubing used for water delivery (Petersen et al. 2014). When particles settle on the bottom in flow-through systems, this may increase the total nanomaterial concentration in the aquaria, even if the suspended concentration remains constant (OECD 2022b).

16. Figure 1 presents a flowchart to decide on the exposure regime. If the aqueous exposure appears most relevant, a pre-test to study the stability of the nanomaterial in the aquarium water needs to be conducted. Section 10345021 provides details on how to perform the pre-test in aquarium water.

Figure 1. Decision tree regarding the most appropriate testing regime based on relevance of the exposure route and nanomaterial suspension stability in aquarium water.



Other recommendations

17. As for soluble chemicals, the feeding regime may influence uptake and elimination of nanomaterials. For instance, the discrepancy in accumulation profile between studies testing the same TiO_2 nanomaterials (NM 105) (Federici et al. 2007; Zhu et al. 2010) could be explained by differences in feeding rates, as well as differences in fish species and sizes used. TG 305 (OECD 2012) indicates a feeding regime depending on the species used, experimental conditions and calorific value of the food (for example for rainbow trout between approximately 1 to 2% of body weight per day). The influence of varying or increasing feeding intake rates on study results have already been discussed in GD 264 (OECD 2017a). BMFs calculated in the studies using lower feeding rates appear lower than those using higher feeding rates.

18. In case a dietary approach is used, feed pellets must be deposited in water and nanoparticles may leach out, leading to variations in the calculated dose ingested by the fish. Section 10345021 indicates how to check the leaching of nanoparticles to water to ensure it is minimal. It is essential, in any case, to calculate an appropriate feeding rate so that animals take up the feed as soon as possible, thus minimising the contact time of the pellets with water. Consequently, it is very important to closely observe animals when feeding to be sure that they eat quickly and that no feed is left in the aquaria. Such leftovers would be indicative of errors in the calculated dose to be eaten by fish. In addition, if the leftovers disperse and nanomaterials are released to water, there could be additional ways of exposure not anticipated initially. Finally, these leftovers can increase the organic matter content of waters, leading to a reduction in water quality that may further influence test results.

19. As indicated in TG 305 (OECD 2012) “the test was originally designed for non-polar organic substances. For this type of substance, the exposure of fish to a single concentration is expected to be sufficient”. However, “if substances outside this domain are tested, or other indications of possible concentration dependence are known, the test should be run with two or more concentrations”. Therefore, in the case of nanomaterials, maximal care should be taken in the decision on the number of concentrations to be tested and the specific concentrations to be used (OECD 2024). Some studies with metal oxide

nanomaterials indicate a potential need for testing at least two different concentrations (Zhu et al. 2010, Abdel-Khalek et al. 2016, Kalman et al. 2023; Connolly et al. 2023). A minimum of two concentrations is advised for testing the bioaccumulation of nanomaterials, except when there is sufficient evidence of a non-concentration dependence.

20. In relation to the extension of the uptake and depuration phases for nanomaterials, there is no specific rule. For some nanomaterials only a short uptake will be needed to reach the steady state[1], together with a short depuration period to eliminate the nanomaterial. For other nanomaterials, longer periods as those indicated in TG 305 (OECD 2012) may be necessary. This is not different from soluble chemicals and TG 305 provides guidance on how to approach the issue of predicting the duration of the uptake and depuration phases for an optimal test set-up *(cf.* paragraphs 37, 38 and 39 of TG 305) (OECD 2012). However, to set the duration of the uptake phase for nanomaterials, the aqueous solubility or the octanol/water partition coefficient of the test substance cannot be used as these properties do not apply to nanomaterials. Besides the noted exceptions, instructions in TG 305 also apply to nanomaterials and must be followed.

21. Solvents and dispersing agents should be avoided (Handy et al. 2012a, OECD 2012). Such agents may artificially change the dispersion and may alter bioavailability and consequently uptake and the bioaccumulation of the nanomaterial in fish. This will hamper comparisons between studies, for instance when results obtained with nanoforms and non-nanoforms of the same substance are required.

22. In addition, and according to TG 305 (OECD 2012), the natural particle content as well as the total organic carbon of the dilution water should be as low as possible to avoid adsorption of the test substance to organic matter. This may reduce nanomaterial bioavailability and therewith result in an underestimation of the BCF. TG 305 prescribes a maximum acceptable value of 5 mg/L for particulate matter (dry matter, not passing a 0.45 µm filter) and 2 mg/L for total organic carbon. These values should also be respected when testing nanomaterials.

23. Salinity, and pH of water in aquaria are all also important factors that can impact both nanomaterial stability and bioavailability. Recommended values for these parameters are indicated in TG 305 (OECD 2012) and need to be followed (TG 305, paragraphs 27, 28 and 53). The water pH value should be within 6.0 and 8.5 at test start and during the test it should remain within a range of ± 0.5 pH units. Potential influence of these small pH changes on nanomaterial dispersions should be detected in regular checks carried out before and during the assay (TG 305, paragraph 53 provides guidance on frequencies of such checks). If it is suspected that pH can strongly influence nanomaterial properties and their dispersions so that the changes allowed in TG 305 (± 0.5 pH units) can provoke strong changes in exposure conditions, detailed checks should be performed prior to the start of the assay. TG 305 mentions the importance of using an appropriate salinity in water especially when marine species are used. High salinity conditions needed in such cases may deeply affect nanomaterial dispersion properties and stability. Therefore, it is crucial to meticulously monitor changes in the size distribution of the nanomaterial when stock dispersions are diluted in waters with higher salinity and maintain heightened vigilance throughout the assay.

[1] It should be noted that nanomaterials do not have a steady-state as understood in colloid chemistry. Here the term is used in accordance with the definition in TG 305 (OECD, 2012), i.e. “A steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20% of each other, and there is no significant increase of C_f in time between the first and last successive analysis.” Several studies have shown that such a plateau in the curve can be reached for nanomaterials as well.

PRE-TEST IN AQUARIUM WATER

Preparation of the stock dispersion

24. The first step for both exposure routes (aqueous and dietary) is the preparation of the stock dispersion. Section 5.5 of GD 317 offers guidance about monitoring of test dispersion stability in the stock suspension and provides a flowchart (Figure 5) to inform development and testing of stock dispersions to be used in preparing test dispersions. The first question is the availability and applicability of a stock media preparation procedure. OECD TG 318 (OECD 2017b) provides such a procedure. Alternatively, the NANoREG dispersion protocol (Booth and Jensen, 2015) can be followed. The NANoREG protocol has demonstrated its usefulness to prepare stable stock dispersions of nanomaterials of different nature. This procedure has been applied to prepare stock dispersions for aquarium water exposures (Pulido-Reyes et al. 2024) and for feed spiking with metal-based nanomaterials (Kalman et al. 2023, 2025).

Stability of the nanomaterial exposure dispersion

25. Prior to the bioaccumulation test, the concentration and size distribution of the nanomaterial over time should be characterized as well as any solute concentrations that result from dissolution of the nanomaterial. The aim would be to determine, before exposing fish to the nanomaterials, the stability of the dispersion over the exposure period when a flow-through system is used and the reproducibility of the exposure suspension over the water renewals in semi-static systems.

26. As explained in paragraph 15, the stability and homogeneity of the dispersion of nanomaterials has to be characterised prior to any animal testing. Guidance Document 317 (OECD 2022b) emphasizes the necessity of monitoring dispersion stability in both the stock suspension and in the test media. According to GD 317 (Section 4.2), performing preliminary tests under similar conditions as those of the full tests is essential to study the stability of dispersions. To minimise the use of test animals, such preliminary tests for using TG 305 (OECD 2012) must be carried out without fish.

27. In general, mass concentration is the variable used to determine stability. Only a deviation within ± 20% of the initial value is allowed. If deviation exceeds this value, the exposure concentration of nanomaterials should be determined at a frequency high enough for calculating average, time-weighted average or geometric mean approaches. Table 3 of GD 317 provides valuable information about nanomaterial and nanomaterial dispersion characterization. As explained above the stability and homogeneity of the nanomaterial dispersion has to be characterized prior to any animal testing. Section 5.5 of GD 317 offers guidance about monitoring of test dispersion stability and underscores that stability determination should include concentration of the nanomaterial in suspension, agglomerate size and dissolution. This characterization must be done in both the stock suspension as well as in the suspensions in the exposure vessels in preliminary tests and then in the full test.

28. Additional guidance on how to characterise the stability of the nanomaterial in the stock suspension and in the aquarium water are described in previous reviews (Handy et al. 2012a, 2012b, Pulido-Reyes et al. 2024), with the notion of achieving stable concentrations during the exposure and minimising the need for water changes in the fish tanks in semi-static systems.

29. As mentioned, it is necessary to conduct some ‘pre-tests’ to determine the stability of the nanomaterial during the exposure period. Attempts at gently mixing the water can enable the dispersion in the fish tanks. Using aeration (i.e., a curtain of air bubbles on one side of the tank) can successfully give continuous mixing in a semi-static exposure system (Federici et al. 2007). In contrast, air stones are advised against as they can break up to release particles into the water. The use of turbines or other flow devices at slow speeds may increase the stability of the suspension as well. High speeds should be

avoided as they can cause laminar flow and shear aggregation (Pulido-Reyes et al. 2024). Again, the precise settings on these devices should be optimised prior to the main study with fish.

METHODOLOGIES FOR FEED SPIKING

Fish feed spiking

30. If a dietary approach for using TG 305 (OECD 2012) with nanomaterials is chosen, it is essential to add nanomaterials to the feed in a way that ensures a reliable and reproducible ingestion by fish of the dose to be tested. According to TG 305, feed spiking should ensure maximum homogeneity and bioavailability of the test substances without influencing the palatability of the spiked feed.

31. Nanomaterials may be added to fish feed following two basic approaches described in TG 305 (OECD 2012). In the first one, nanomaterials can be added as a dry powder and blended with the other dry ingredients (fish meal, mineral supplements, etc.) prior to binding all the ingredients with a small amount of water (or corn oil as appropriate) and pressing/extruding the feed into pellets. This approach has the advantage of homogeneously incorporating the test material among the other components within the food pellets. It, however, requires expertise in feed formulation and pellet production, and to have availability of a system for feed production. This is not common in most laboratories. Therefore, the second possibility may be more feasible and easier. It consists of preparing a dispersion of nanomaterials in a liquid (water, ethanol or oil). This is then used to soak the pellets of an existing commercial fish feed or, it is sprayed onto the feed pellets to give the required dietary concentration. In either case, care should be taken not to make the feed too wet. It can be beneficial to top-coat the nanomaterial-coated feed with a layer of 10% gelatine solution and allow this to dry. This practice could improve the nanomaterials staying on the food pellets when added to the aquarium water. It may also improve the palatability by hiding the taste of the test material when the feed is ingested by the fish. These methods are allowed in TG 305 (OECD 2012) and are discussed in detail for nanomaterials of different nature in Handy et al. (2018) and Connolly et al. (2023).

32. Regardless of the approach adopted, the stock suspension of the nanomaterial in the appropriate liquid (water, ethanol or oil) should be characterised (see also Section 4.2 and Table 3 in GD 317). The primary size, particle size distribution, zeta potential, concentration and dissolution should be provided. Ideally, stock suspensions should be prepared each time that feed is spiked. In any case, before applying it, it is necessary to ensure that original properties are maintained.

33. According to TG 305 (OECD 2012), after spiking feed with the stock suspension, the concentration of the nanomaterial in the feed must be measured at least at the start and end of the feeding period (see Chapter 6). The homogeneity of the spiking should be assessed. For that, pellets should be sampled for the corresponding analyses (see Figure 2). Ideally, each food pellet should contain the same amount of the test substance. An appropriate amount of feed should be administered that is quickly and totally consumed, allowing the calculation of the ingested dose. The goal here is to ensure that fish are exposed to the same concentration during the uptake phase and that only a dietary exposure occurs without remains of feed in the fish tanks that could leach nanomaterials into the aquarium water.

34. As for other substances, the concern of nanomaterials leaching from the feed into the water during the feeding time should also be addressed. This can be done by doing some preliminary leaching experiments in the aquarium water without fish to determine if the pellets remain stable (see Section 5.2). In practice, a stable, palatable feed, and/or a gelatine top-coated feed would be ingested quickly (seconds to a minute) with negligible risk of leaching and loss of ingested dose. Odour and visual checks should be done for rancidity or changes in texture/colour of the feed. Rancidity is usually caused by oxidation of lipids, with subsequent depletion of the vitamins (antioxidants) in the feed during storage. It makes the food

unpalatable as well as causing oxidative damage to the tissues of fish if ingested (e.g., Baker et al. 1998). This would be especially a concern for nanomaterials made of known oxidising substances (e.g., Cd). Texture and colour are also important, and for example, high doses of carbon nanotubes can make the feed appear black and make the pellets hard (e.g., Fraser et al. 2011). Importantly, the feed should retain its buoyancy properties according to the needs of the species (e.g., floating feed for carp or trout). In addition, measuring chemical changes in the composition of the feed, or the state of the nanomaterials (i.e. agglomeration, ion release) in/on the pellets can be challenging. These kinds of problems could be avoided by preparing the feed in small batches for immediate use that week, or even daily. For nanomaterials, the daily preparation is recommended to minimise any change of the nanomaterial although this will result in the need for additional concentration measurements. Feed must be stored dry, cool, and in the dark. Freezing the food (i.e., to arrest any rancidity reactions) can extend its storage for a few weeks, but slow defrosting of rations at room temperature would be necessary prior to any feeding session. Re-freezing of feed should absolutely be avoided. If freezing and thawing the dosed-feed is part of the test protocol, the effect of this process on the nanomaterial structure and distribution following dispersal in the test system should be evaluated.

35. As an example, a detailed and recommended protocol to prepare spiked diet using a nanomaterial aqueous stock suspension is described in Kalman et al. (2023, 2025). This protocol was used by three different laboratories in an interlaboratory study and the methodology and results are presented in Annex Annex 1. shows an overview of the process. Briefly, a stock suspension is prepared in MilliQ water, ethanol or oil, as appropriate (see section 4.1). The stability of the nanomaterial is determined by size distribution, ion release and concentration in three replicates. An appropriate aliquot of the stock suspension is directly added to the feed to ensure homogeneous concentration in each pellet and avoid losses to the tubes (0.2 mL/g in this example, added in two aliquots of half total volume needed). Following TG 305 (OECD 2012), a sufficient mass of feed should be spiked in one preparation and used for the duration of the uptake phase. However, as already mentioned, it is advisable to prepare daily fresh stock suspensions of the nanomaterial and to check for the reproducibility of the process (at least in triplicate). For this purpose, the whole process of spiking should be repeated three times from the same suspension and the reproducibility of the process using different suspensions should be assessed for three different stock suspensions (triplicate). Spiked feed is dried overnight at room temperature and fed to fish. Homogeneity, recovery and reproducibility of spiking is assessed from three aliquots of 100 mg spiked feed.

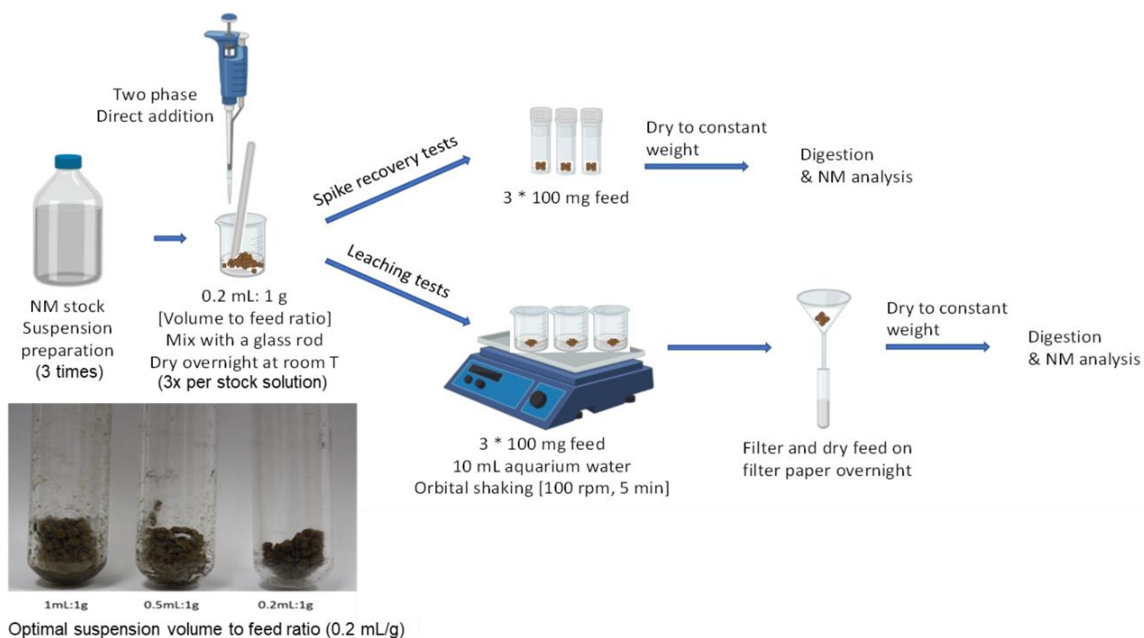
36. These approaches, based on the use of stock suspensions in water, appear to be the simplest ones and guarantee good results (Kalman et al. 2023, 2025). When nanomaterials are provided as a powder, a stock suspension can be generated (see section 4.1). These stock suspensions or nanomaterials provided as suspensions may need to be diluted to achieve the appropriate concentration in feed. Besides water, other media or liquids can be used to obtain the initial suspension before being soaked on the pellets. Suspensions in olive oil or sunflower oil can also be used (Bermejo-Nogales et al. 2021). With oil being more viscous than water, these suspensions may be more difficult to manage, and special consideration must be given to possible losses due to material that remains adhered to the container walls. Ethanol or other organic solvents indicated in TG 305 (OECD 2012) may also be tried in case that problems arise with water or oils. A detailed study on the stability of the stock suspension is mandatory to ensure a homogeneous distribution of the nanoparticles on the feed. In any case, it is essential to ensure that appropriate doses of nanomaterials appear on or in the pellets (see Chapter 6).

Leaching test

37. As mentioned in section 1.2, it is essential, after preparing the pellets spiked with the nanomaterial, to perform a leaching test (see Figure 2). This should ensure that the amount of released nanoparticle is minimal and does not imply a significant variation in the calculated ingested dose.

38. Potential release of nanomaterial from spiked feed to the water is determined in triplicates, following 5 min immersion in aquarium water (100 mg feed, 10 mL water and agitation on an orbital shaker at 100 rpm). An appropriate technique for the nanomaterial under study (see Chapter 6) is used to measure the nanomaterial concentration. The reproducibility of this protocol was confirmed in three independent laboratories. Results are presented in Annex 1.

Figure 2. Feed spiking methodology (including reproducibility and recovery checks) and leaching test (Kalman et al. 2023). Stock suspension volume is added in two aliquots of half total volume needed. To assess the reproducibility of spiking, three beakers should be prepared from the stock suspension. To ensure the reproducibility of the process, it should be assessed in triplicate preparing three different stock suspensions. Leaching should be assessed from one beaker in triplicate.



METHODOLOGIES FOR DETERMINATION OF NANOMATERIALS AND THEIR TRANSFORMED FORMS IN AQUARIUM WATER, FISH FEED AND FISH TISSUES

39. This topic strongly links to the Guidance Document on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies (expected to be published in 2026). It is advised to consult that Guidance Document (once published) for further guidance on determination of nanomaterials in complex matrices.

Sampling methods

40. Sampling from the water column will be required when bioaccumulation is assessed via aqueous exposure. Aqueous sampling is also needed in pre-tests for stability and leaching studies. In both circumstances samples should be taken from the midpoint of the water column (in the x, y and z directions) to avoid the vessel walls and the bottom. This represents exposure to the suspended and stable material. Although the possible sorption of nanomaterials to collection vessels/pipettes is difficult to predict, it is essential to avoid the use of containers that could absorb these materials, and the possibility of sorption

should be considered already in pre-tests. The frequency of sampling will be based on results of stability pre-tests (see also OECD TG 318; OECD 2017b). This should ensure a sampling frequency to be sufficiently robust to allow a consistent characterisation of the material exposure (e.g. samplings at a minimum every 24 h and directly after a water renewal). During pre-tests, samplings as early as 6 h could already serve to identify highly unstable materials. For metal-based materials, samples will also need to be analysed for dissolved and particulate forms. These samples must be analysed immediately (e.g. by sp-ICP-MS), or at least fractions (particulates vs. dissolved) should be separated immediately. Multiple techniques to achieve separation are possible (e.g. field flow fractionation (FFF), ultracentrifugation or the use of centrifugal filtration units).

41. As described in paragraphs 30-36, spiked feed should be sampled at least at the start and end of the experiment. However, to ensure a consistent exposure concentration, it is recommended to prepare spiked feed daily (with appropriate checks on concentrations).

42. For the sampling method and frequency of fish and (where relevant) fish tissues, TG 305 (OECD 2012) should be followed. For further assessment of nanomaterial concentrations in the tissues the following sections provide further guidance.

Storage of samples

43. When the hydrodynamic diameter of the nanomaterial in the stock suspension or in the aquarium water suspension will be measured, suspensions should be maintained at the experimental temperature conditions and measured immediately. However, when the concentration of the nanomaterial is going to be analysed, samples not analysed immediately should be stored under appropriate conditions to avoid any potential transformations or changes in material properties from those at the time of sampling. This applies to aquarium water, feed and fish tissue samples. For each type of sample, optimum storage conditions should be defined.

44. Fish tissues should be frozen (e.g. snap freezing in liquid nitrogen or direct storage at -20 °C or -80 °C is appropriate). This will preserve the tissue specimen as quickly and closely as possible to the *in vivo* state. Water samples can be stored at 4 °C. Spiked feed samples can also be stored under refrigeration (at 4 °C) in a sealed airtight container. Potentially photoreactive materials should be protected from light. The low temperature is necessary to limit any fungal/bacterial growth in the feed or rancidity. However, both water and feed samples stored at 4 ºC must be analysed as soon as possible, preferably within a week. At this temperature, transformation of organic matter may still take place, provoking changes that would affect analytical results. By limiting the storage time such transformation can be minimised. When using total elemental analysis for metal-based nanoparticles or carbon-based nanomaterials, agglomeration or dissolution will not influence the analysis.

Sample digestion/processing

45. Before chemical analyses of the substances of interest, it will be probably necessary to include steps for sample processing to break down the material or remove the organic material prior to analysis. This will be certainly necessary in the case of feed pellets and fish tissues. For metal-based nanomaterials, these procedures are usually performed using a strong nitric acid (HNO_3) or similar, however in particular cases combinations of other acids and/or hydrogen peroxide (H_2O_2) may be required depending on the specific matrix and particular analytes. When the particulate nature of the nanomaterial should be preserved, these methods cannot be used (see also section 6.4). In general, optimisation and validation are necessary to ensure recovery is satisfactory. OECD Test Guideline Programme Project 1.10, mentioned before, will give additional and detailed information on determination of concentrations of nanoparticles in biological samples once published as OECD GD.

Analytical methods

46. There exist important analytical challenges for measuring nanomaterials, especially in fish feed and tissues (Handy et al 2018). Techniques for detection and definitive identification of nanomaterials in samples exist and are constantly advancing, so that they may solve present and future challenges in this field.

47. The fundamental basis for the bioaccumulation assay relies on quantitative analysis. Therefore, only analytical techniques that have sufficient quantitative power of determination can be considered in the context of the application of TG 305 (OECD 2012). Also, according to TG 305, samples should be analysed by an established and validated method and its quality (specificity, accuracy, precision and reproducibility) should be demonstrated.

48. Information should be provided about the method's limit of quantification, percentage of recovery and reproducibility, as well as potential interferences and any analytical variability in the specific sample matrix (water, feed, fish). The current best practice is to involve a series of spike and recovery tests that include: (i) a pristine nanomaterial suspension for reference, (ii) procedural blanks and blanks spiked with the nanomaterial of interest, (iii) control fish tissue/feed (spiked for analysis, and not spiked), and (iv) if possible, a certified reference material. However, certified reference materials of fish tissue containing certified amounts of nanomaterial are not currently available. Selection of quantification method will depend on the nanomaterial composition (e.g. presence of metallic elements) and intrinsic/unique characteristics (thermal, spectroscopic, fluorescent, magnetic properties) that could aid detection and quantification.

49. In case the nanomaterial tested contains a natural occurring material in the sample matrix (such as a ubiquitous metal or carbon), the analytical technique used must be sensitive enough to allow correction for this background. This may present some limitations for the application of this test. For example, the high carbon backgrounds in feed and fish samples currently hamper quantification of carbon-based nanomaterials in these matrices. Traditional chemical analysis such as ICP-MS for metals, and sometimes coupled with various high performance liquid chromatography (HPLC) based techniques for sample separation, can be useful for the measurement of the total amount of the metal (assumed from the nanomaterial) that has been accumulated. These methods cannot distinguish the accumulated nanomaterial as total metal from high background levels. Control groups (non-treated animals) are therefore essential in the experimental design to enable some quantification of the naturally occurring chemical background in the tissues. In fish, like in other animals, it is the bulk electrolytes (Na, K, Ca, Mg) that show relatively high levels, and may present a detection problem for, e.g., a hydroxyapatite nanomaterial that is also rich in Ca. Fish tissues also have a high background level of iron (problematic for zero valent ion particle detection), and sometimes zinc in certain tissues.

50. Radiolabelling is permitted under TG 305 (OECD 2012) and can be used to facilitate the analysis of nanomaterials in water, feed and fish tissues, particularly when there are high background levels of the element of interest. However, any significant modification in a nanomaterial's physical characteristics caused by the radiolabelling/irradiation process should be assessed. Different radioisotopes can be used (e.g. ^{14}C or ^{125}I), the most common being ^{14}C.

51. For metal-containing nanomaterials, the total metal concentration in samples can be analysed by ICP-OES or ICP-MS after chemically digesting the pellets in strong acid. For these techniques, standardised procedures are available and can be consulted, provided the sample is diluted to reduce the concentration of the acid (e.g., ISO standards for determination of selected elements in water; ISO, 2023). In situations where more detailed information is needed about the amount of total metal content and the proportion of nanoparticle compared to the dissolved fraction, single particle ICP-MS (spICP-MS) could be applied. Routine ICP-MS cannot determine the presence of particles, so the spICP-MS technique should be used for the latter. The spICP-MS approach has some current technical limitations: it only works for

metal-containing particles and only a few types of metals have been extensively studied (e.g., silver nanoparticles, Clark et al. 2021). Furthermore, spICP-MS generally is not able to detect primary particles of < 20 nm in diameter and using the technique for particles made of multiple metals is challenging. For tissues, the extraction method or tissue digestion should not alter the particles (e.g., cause dissolution that may alter particle size). There is an ISO method that can be followed for liquid suspensions (ISO 2024), although it does not specifically have the matrices of extracted tissue samples in mind and is not particle-specific. However, additional information on such detailed methods for tissue samples will appear in the mentioned Guidance Document on the determination of concentrations of nanoparticles in biological samples for (eco)toxicity studies (expected to be published in 2026).

52. Electron microscopy can determine the presence or absence of a nanomaterial, and even confirm the primary particle size and/or the presence of coating (OECD TG 125 (OECD 2023). However, electron microscopy has not yet been successfully applied to the routine quantitative determination of nanomaterials in animal feed or tissues although some attempt to develop such methods have been made (e.g. Muhfeld et al, 2007). In addition, the method has some more general limitations as outlined in OECD TG 125 (OECD 2023), including that the procedure of visualizing and counting particles currently often relies on manually examination (although automated methods are under development). Methods that attempt to give quantitative information on material elemental composition, such as X-ray fluorescence (XRF) electron microscopy, can sometimes be used to confirm the presence of a nanomaterial in a mixture of naturally occurring particles, but the challenge remains to find the nanomaterial in the sample in the first place in order to make such measurements.

53. For determination of nanomaterials in aquarium water, samples are taken from the water-column of the tanks as previously indicated. For metallic-based nanomaterials in aqueous media, the routine techniques used for analysis are ICP-OES or ICP-MS as indicated before. The coupling of multiple techniques to achieve separation and purification is possible. For example, using field flow fractionation (FFF) for separation of particle fractions, followed by chemical analysis by spICP-MS for Ag-containing particles, has been applied in pure waters spiked with bovine serum albumin (Mitrano et al. 2012). However, FFF can be time consuming, and advances in spICP-MS have been made to directly measure the size distribution and particle number concentration of metallic-based nanoparticles in natural waters (Bevers et al. 2023; Wang et al. 2024).

54. For carbon-based nanomaterials like fullerenes (Isaacson et al. 2009), graphene-based materials (Moles et al. 2024), carbon nanotubes (CNTs) (Cerrillo et al. 2015, Pulido-Reyes et al. 2024), aluminosilicates or others (Pulido-Reyes et al. 2024), UV-vis spectrometry can be used to determine the concentration of these nanomaterials in the aquarium water, although this technique has limitations in determining low concentrations. Total carbon is a routine water quality measurement, and for carbon-based materials the total carbon in the treatments compared to controls could be used to estimate the expected nanomaterial concentration. However, as indicated above, the high carbon background in animal feed, sediments, and soils currently prevent this method of quantification in complex matrices.

BCF AND BMF DERIVATION

55. It should be noted that underlying mechanisms of uptake and elimination of nanomaterials are different from those for solutes (i.e.: active uptake versus passive diffusion, respectively; Handy et al. 2018, see also Li et al. 2008). Nevertheless, concentrations in fish may show accumulation and sometimes the appearance of steady state kinetics. Derivation of apparent BCF and BMF values for nanomaterials may thus be possible.

56. Calculation of a BCF or BMF is based on uptake and elimination processes (TG 305, Annex 5, OECD 2012). For soluble chemicals these processes are generally described ‘reasonably’ well by first

order kinetics in a simple two-compartment/two-parameter model (GD 264, OECD 2017a). In many cases, the kinetic BCF (BCF_K) will be the preferred measure of bioconcentration in the aqueous exposure test, but whenever possible, both BCF_K and the steady-state BCF (BCF_{SS}) should be determined and reported (GD 264, OECD 2017a).

57. Estimation of BCF_{SS} is relatively straight-forward, also for nanomaterials, by calculating the ratio of concentrations in the fish and in the water when the steady-state is reached (TG 305, Annex 5). However, as stated in GD 264, BCF_{SS} can also show some disadvantages. It does not use all data generated in the experiment, so that increases in the dilution and loss processes may not have been taken into account. In addition, steady-state may not be reached within the standard duration of the experiment. For this and other reasons, GD 264 highlights that it is desirable to always report the BCF_K as well as BCF_{SS}. As underlying mechanisms of uptake and elimination of nanomaterials are different from those for solutes, this advice should be followed for nanomaterials as well to allow identification and interpretation of any differences between BCF_K and BCF_{SS}.

58. BCF_K can be calculated by the sequential and simultaneous fit methods. Both are based on the same principles of first order kinetics. The difference is that simultaneous fit methods allow a better recognition of overestimations providing more information on the quality of data and the adjustment to the model (standard errors and confidence intervals for each of the estimated parameters) (see also GD264, Annex 3 for discussion on the differences). These additional insights on the quality of the data may be useful for interpretation of the results for nanomaterials and thus the simultaneous fit methods are recommended for nanomaterials.

59. As indicated in TG 305 (OECD 2012) fish growth as well as lipid content may influence the calculated values for BMF and BCF and correction for both may be appropriate. Growth and lipid content are dependent on the fish used and will (in principle) not be influenced by the test substance. For this reason, approaches to correct for these influences for soluble chemicals are assumed to be applicable for nanomaterials as well. These approaches are described in TG 305 and its accompanying GD 264 (OECD 2017a).

60. For soluble chemicals, uptake processes are generally driven by diffusion processes, while for nanomaterials this is not the case (Handy et al. 2018). This may raise questions on the validity of calculations that are assuming first order kinetics in a simple two-compartment/two-parameter model, although uptake and depuration of nanomaterials may still be described 'reasonable' well with such a model. To test that hypothesis, data from accumulation experiments with nanomaterials were used in calculations according to GD 264 (OECD 2017a) (facilitated by the accompanying R-package, '*bcmfR*') to assess whether first order kinetics models can describe uptake and depuration of nanomaterials as well. Data used were generated in the Gov4Nano project in which several bioaccumulation studies were performed with CuO (both rod-shaped and spherical nanomaterials) (Kalman et al. 2023) and CdTe quantum dots (either carboxyl- or polyethylene glycol-coated) (Kalman et al. 2025), with both dietary and aqueous exposures. The examples in Figure 3 suggest that for fish exposed to nanomaterials through water, uptake and depuration of these nanomaterials can still be described with the models used for soluble chemicals. Similarly, after dietary exposure, the models show 'reasonable' fits with the concentrations in fish during the depuration phase (Figure 4.).

Figure 3. Left, orange lines: Uptake and depuration in rainbow trout (*Oncorhynchus mykiss*) during/after aqueous exposure to rod-shaped copper oxide nanoparticles (based on Box-Cox transformations, see GD 264). Right, blue lines: Uptake and depuration in rainbow trout during/after aqueous exposure to carboxyl-coated cadmium-telluride quantum dots (COOH-CdTe) (based on non-transformed data, see GD 264, OECD 2017a). CFish (ugX/kgW): concentration in fish (µg substance/kg fish); dotted lines indicate 95% confidence intervals.

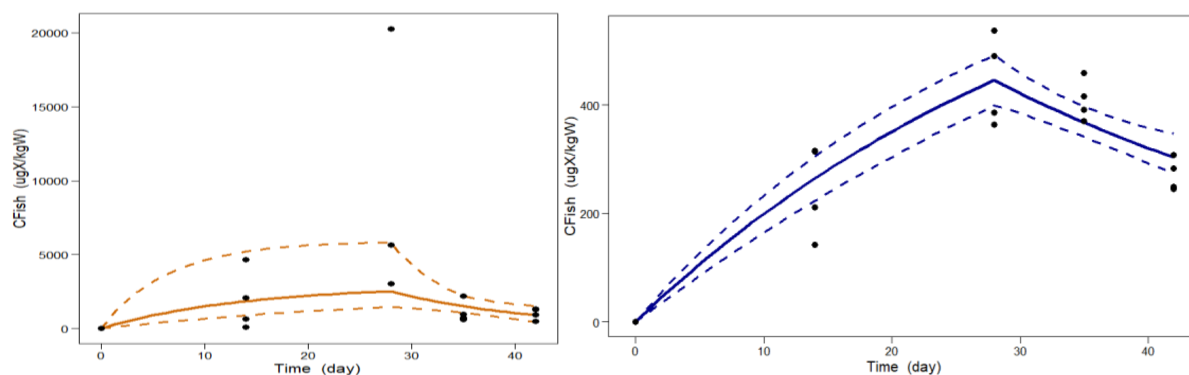
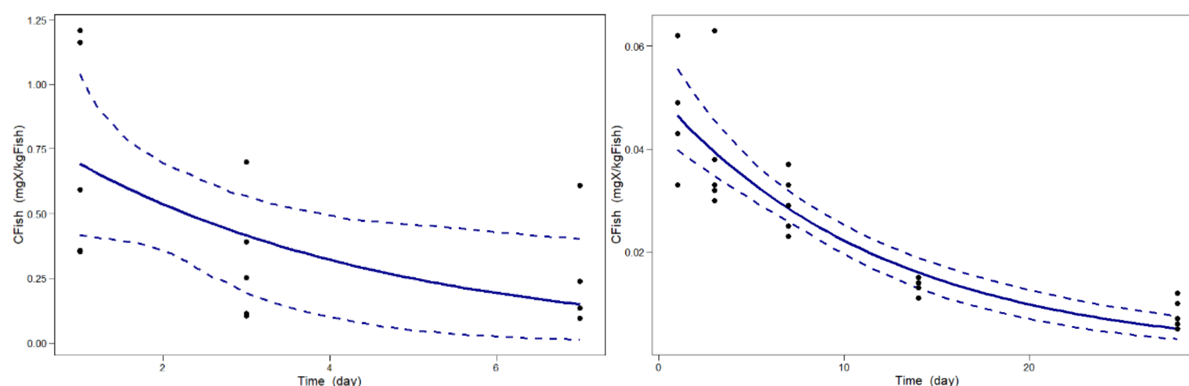


Figure 4. Left: Depuration in rainbow trout (*Oncorhynchus mykiss*) after dietary exposure to rod-shaped copper oxide nanoparticles. Right: Depuration in rainbow trout after dietary exposure to carboxyl-coated cadmium-telluride quantum dots (COOH-CdTe). Plots are based on non-transformed data (see GD 264, OECD 2017a). CFish (ugX/kgW): concentration in fish (µg substance/kg fish); dotted lines indicate 95% confidence intervals.



61. When the steady state and a good depuration is reached, the results from these assays showed very similar BCF values when calculated at the steady state or by the different kinetic methods (sequential and simultaneous fit methods). The same was observed for the derived BMF when calculated on the basis of kinetic or steady state methods. When this is not the case and when data suggest that deviations from first-order kinetics may exist, a careful interpretation of the best model to obtain these results is needed. This is not different for soluble chemicals.

62. While these are promising results, it is worth noting that these examples are based on metal-based nanomaterials, where solubility and metal-related diffusion processes may play a role. However, there are other examples where such processes are not relevant and the steady state was also reached. These examples included a BCF at the steady state for multiwall carbon nanotubes (MWCNT) (Maes et al. 2014; Cano et al. 2018) and for Se nanoparticles *versus* selenite (Li et al. 2008). Unfortunately, the kinetic values were not calculated in these studies, avoiding a comparison between both results.

63. Li et al. (2008) performed a comparison study on the bioaccumulation of Se nanoparticles versus selenite and found differences in the kinetics of both compounds. A higher bioaccumulation was seen for Se nanoparticles compared to the non-nanoform selenite clearly demonstrating differences in kinetics of nanoparticles versus ions.

CONVERSION OF BMF TO BCF FOR BIOACCUMULATION ASSESSMENT

64. Many regulatory risk assessments rely on BCF values rather than BMF values. For this reason, GD 264 (OECD 2017a) discusses pros and cons for potential methods to convert BMF to BCF. Many of these methods, however, rely on the octanol-water partition coefficient, which renders them not applicable to nanomaterials. A lack of data makes it difficult to find correlations between BMF and BCF values (even more so than for non-nanomaterials).

65. In general, the recommendations for nanomaterials are similar to those for non-nanomaterials given in GD 264 (paragraph 297). That means that where BMF values for nanomaterials are converted to a BCF value, such BCF values should be treated with caution, especially where such values are close to a regulatory criterion.

References

Abdel-Khalek, A.A., Badran, S.R., Marie, M.A. (2016). Toxicity evaluation of copper oxide bulk and nanoparticles in Nile tilapia, *Oreochromis niloticus*, using hematological, bioaccumulation and histological biomarkers. Fish Physiol. Biochem. 42, 1225–1236.

Baker, R.T.M., Handy, R.D., Davies, S.J. and Snook, J.C. (1998). Chronic dietary exposure to copper affects growth, tissue lipid peroxidation, and metal composition of the grey mullet, *Chelon labrosus*. Marine Environmental Research, 45(4-5), 357-365.

Bermejo-Nogales, A., Rucandio, I., Connolly, M., Fernández-Cruz, M.L., Navas, J.M. (2021) Preparation of feed with metal oxide nanoparticles for nanomaterial dietary exposure to fish and use in OECD TG 305. MethodsX, 8:101413; doi: 10.1016/j.mex.2021.101413

Bevers, S.G., Smith, C., Brown, S., Malone, N., Fairbrother, D.H., Goodman, A.J. and Ranville, J.F. (2023). Improved methodology for the analysis of polydisperse engineered and natural colloids by single particle inductively coupled plasma mass spectrometry (spICP-MS). *Environmental Science: Nano*, *10*(11), pp.3136-3148.

Booth, A., Jensen, K.A. (2015). SOP NANoREG-ECOTOX. Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media. Preparation and physicochemical characterisation of manufactured nanomaterials (MNMs) in environmental fate and ecotoxicity exposure media. Dispersion Standard Operating Procedure. Version 6. https://safenano.re.kr/download.do?SEQ=177

Cano, A.M., Maul, J.D., Saed, M., Irin, F., Shah, S.A., Green, M.J., French, A.D., Klein, D. M., Crago, J., Canas-Carrell, J.E. (2018). Trophic transfer and accumulation of multiwalled carbon nanotubes in the presence of copper ions in *Daphnia magna* and fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 52, 794–800.

Cerrillo, C., Barandika, G., Igartua, A., Areitioaurtena, O., Marcaide, A., Mendoza, G. (2015). Ecotoxicity of multiwalled carbon nanotubes: Standardization of the dispersion methods and concentration measurements. Environ Toxicol Chem. 34:1854–1862.

Clark, N.J., Boyle, D., Eynon, B.P. and Handy, R.D. (2019). Dietary exposure to silver nitrate compared to two forms of silver nanoparticles in rainbow trout: bioaccumulation potential with minimal physiological effects. *Environmental Science: Nano*, *6*(5), pp.1393-1405.

Clark, N.J., Clough, R., Boyle, D. and Handy, R.D. (2021). Quantification of particulate Ag in rainbow trout organs following dietary exposure to silver nitrate, or two forms of engineered silver nanoparticles. *Environmental Science: Nano*, *8*(6), 1642-1653.

Connolly, M., Martínez-Morcillo, S., Kalman, J., Navas, J.M, Bleeker, E., Fernández-Cruz, M.L. (2023). Considerations for bioaccumulation studies in fish with nanomaterials. Chemosphere, 312:137299; DOI: 10.1016/j.chemosphere.2022.137299

Cornelis, G. “Fate descriptors for engineered nanoparticles: the good, the bad, and the ugly,” Environmental Science: Nano, vol. 2, pp. 19-26, 2015.

ECHA 2022: https://echa.europa.eu/documents/10162/17224/appendix_r7a_nanomaterials_en.pdf/ Guidance on information requirements and chemical safety assessment Appendix R7-1 for

nanomaterials applicable to Chapter R7a Endpoint specific guidance. https://echa.europa.eu/documents/10162/17224/appendix_r7a_nanomaterials_en.pdf

Federici, G., Shaw, B. J. and Handy, R. D. (2007). Toxicity of titanium dioxide nanoparticles to rainbow trout, (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. Aquatic Toxicology, 84, 415-430.

Fraser, T. W. K., Reinardy, H. C., Shaw, B. J., Henry, T. B., Handy, R. D. (2011). Dietary toxicity of single-walled carbon nanotubes and fullerenes (C60) in rainbow trout (*Oncorhynchus mykiss*). Nanotoxicology, 5(1), 98-108.

Giese, B., Drapalik, M., Zajicek, L., Jepsen, D., Reihlen, A., Zimmermann, T. (2020). Advanced materials: Overview of the field and screening criteria for relevance assessment. Umweltbundesamt Texte 132/2020.
https://www.umweltbundesamt.de/en/publikationen/advanced-materials-overview-of-the-field-screening.

Handy, R. D., Cornelis, G., Fernandes, T., Tsyusko, O., Decho, A., Sabo-Attwood, T., Metcalfe, C., Steevens, J.A., Klaine, S.J., Koelmans, A.A., and Horne, N. (2012a). Ecotoxicity test methods for engineered nanomaterials: Practical experience and recommendations from the bench. Environmental Toxicology and Chemistry 31(1), 15-31.

Handy, R. D., van den Brink, N., Chappell, M., Mühling, M., Behra, R., Dušinská, M., Simpson, P., Ahtiainen, J., Jha, A. N., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T. F. and Riediker, M. (2012b). Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: What have we learnt so far? Ecotoxicology, 21, 933-72.

Handy, R. D. Ahtiainen, J., Navas, J. M., Goss, G., Bleeker, E. A. J. and von der Kammer, F. (2018). Proposal for a tiered dietary bioaccumulation testing strategy for engineered nanomaterials using fish. Environmental Science: Nano, 5, 2030-2046.

Isaacson, C.W., Kleber, M. and Field, J.A. (2009). Quantitative analysis of fullerene nanomaterials in environmental systems: a critical review. Environmental Science and Technology, 43, 6463-6474.

ISO (2023). ISO 17294-2:2023 – Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS) - Part 2: Determination of selected elements including uranium isotopes. https://www.iso.org/standard/82245.html

ISO (2024). ISO/TS 19590:2024 – Nanotechnologies — Characterization of nano-objects using single particle inductively coupled plasma mass spectrometry. https://www.iso.org/standard/82209.html

Johnston, B.D., Scown, T.M., Moger, J., Cumberland, S.A., Baalousha, M., Linge, K., van Aerle, R., Jarvis, K., Lead, J.R., Tyler, C.R. (2010). Bioavailability of nanoscale metal oxides TiO2, CeO2, and ZnO to fish. Environ. Sci. Technol. 44, 1144–1151.

Kalman, J., Connolly, M., Abdolahpur-Monikh, F., Fernández-Saavedra, R., Cardona-García, A.I., Conde-Vilda, E., Martínez-Morcillo, S., Peijnenburg, W.J.G.M., Rucandio, I.**,** Fernández-Cruz, M.L. (2023) Bioaccumulation of CuO nanomaterials in rainbow trout: Influence of exposure route and particle shape. Chemosphere, 310:136894 ; DOI: 10.1016/j.chemosphere.2022.136894

Kalman, J., Connolly, M., Fernández-Saavedra, R., Cardona-García, A.I., Conde-Vilda, E., Handy, R., Rucandio, I.**,** Fernández-Cruz, M.L. (2025) Bioaccumulation of CdTeQDs nanomaterials in rainbow trout: Influence of exposure route and coating. (Under preparation)

Kamunde, C., Clayton, C. and Wood, C.M. (2002). Waterborne vs. dietary copper uptake in rainbow trout and the effects of previous waterborne copper exposure. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *283*(1), R69-R78.

Laycock, A., Clark, N.J., Clough, R., Smith, R. and Handy, R.D. (2022). Determination of metallic nanoparticles in biological samples by single particle ICP-MS: a systematic review from sample collection to analysis. *Environmental Science: Nano*, *9*(2), 420-453.

Li, H., Zhang, J., Wang, T., Luo, W., Zhou, Q., Jiang, G. (2008). Elemental selenium particles at nano-size (Nano-Se) are more toxic to Medaka (*Oryzias latipes*) as a consequence of hyper-accumulation of selenium: a comparison with sodium selenite. Aquat. Toxicol. 89, 251–256.

Maes, H.M., Stibany, F., Giefers, S., Daniels, B., Deutschmann, B., Baumgartner, W., Schaffer, A. (2014). Accumulation and distribution of multiwalled carbon nanotubes in zebrafish (*Danio rerio*). Environ. Sci. Technol. 48, 12256–12264.

Mitrano, D. M., Barber, A., Bednar, A., Westerhoff, P., Higgins, C. P. and Ranville, J. F. (2012.) Silver nanoparticle characterization using single particle ICP-MS (SP-ICP-MS) and asymmetrical flow field flow fractionation ICP-MS (AF4-ICP-MS). Journal of Analytical Atomic Spectrometry. 27(7), 1131-42.

Molés, G., Connoly, M., Valdehita, A., Pulido-Reyes, G., Fernández-Cruz, M.L., Flahaut, E., Navas, J.M. (2024). Testing the aquatic toxicity of 2D few-Layer Graphene Inks Using Rainbow Trout (Oncorhynchus mykiss): In Vivo and In Vitro Approaches to Support an SSbD Assessment. Toxics 12, 97 DOI: 10.3390/toxics12020097

Muhlfeld, C.; Rothen-Rutishauser, B.; Vanhecke, D.; Blank, F.; Gehr, P.; Ochs, M. Visualization and quantitative analysis of nanoparticles in the respiratory tract by transmission electron microscopy. Part. And Fib. Tox. 2007, 4. doi:10.1186/1743-8977-4-11.

OECD (2012), Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris, https://doi.org/10.1787/9789264185296-en..OECD (2014), . *Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines*, Series on the Safety of Manufactured Nanomaterials, No. 40, OECD Environment, Health and Safety Publications,ENV/JM/MONO(2014)1, ,, https://one.oecd.org/document/ENV/JM/MONO(2014)1/en/pdf

OECD (2017a), *Guidance Document on Aspects of OECD TG 305 on Fish Bioaccumulation*, OECD Series on Testing and Assessment, No. 264, OECD Publishing, Paris, https://doi.org/10.1787/c25ad843-en. OECD (2017b), Test No. 318: Dispersion Stability of Nanomaterials in Simulated Environmental Media, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris, https://doi.org/10.1787/9789264284142-en. OECD (2021), *Guidance Document for the Testing of Dissolution and Dispersion Stability of Nanomaterials, and the Use of the Data for Further Environmental Testing and Assessment*, OECD Series on Testing and Assessment, No. 318, OECD Publishing, Paris, https://doi.org/10.1787/f0539ec5-en.

OECD (2022a). *Advanced Materials: Working Description*, OECD Series on the Safety of Manufactured Nanomaterials and other Advanced Materials, OECD Publishing, Paris, https://doi.org/10.1787/4b5ba38d-en. OECD (2022b). *Guidance Document on Aquatic and Sediment Toxicological Testing of Nanomaterials*, OECD Series on Testing and Assessment, No. 317, OECD Publishing, Paris, https://doi.org/10.1787/08905c99-en.

OECD (2023). *Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials*, OECD Guidelines for the Testing of Chemicals, Section 1, OECD Publishing, Paris. https://doi.org/10.1787/af5f9bda-en

OECD (2024), *A Tiered Approach for Reliable Bioaccumulation Assessment of Manufactured Nanomaterials in the Environment Whilst Minimising the Use of Vertebrate Testing*, OECD Series on the Safety of Manufactured Nanomaterials and other Advanced Materials, OECD Publishing, Paris, https://doi.org/10.1787/2f16cda4-en.

Petersen, E. J., Henry, T. B., Zhao, J., MacCuspie, R. I., Kirschling, T. L., Dobrovolskaia, M. A., Hackley, V., Xing, B. and White, J. C. (2014). Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. Environmental Science and Technology. 48(8), 4226-4246.

Petersen, E., Barrios, A.C., Bjorkland, R., Goodwin Jr, D.G., Li, J., Waissi, G. and Henry, T. (2023). Evaluation of bioaccumulation of nanoplastics, carbon nanotubes, fullerenes, and graphene family materials. Environment International, 173, p.107650.

A. Praetorius, N. Tufenkji, K.-U. Goss, M. Scheringer, F. von der Kammer and M. Elimelech, “The road to nowhere: equilibrium partition coefficients for nanoparticles,” Environmental Science: Nano , vol. 1, pp. 317-323, 2014.

G. Cornelis, “Fate descriptors for engineered nanoparticles: the good, the bad, and the ugly,” Environmental Science: Nano, vol. 2, pp. 19-26, 2015.

Pulido-Reyes G, Moreno-Martín G, Gómez-Gómez B, Navas JM, Madrid-Albarrán Y, Fernández-Cruz ML (2024). Fish acute toxicity of nine nanomaterials: Need of pre-tests to ensure comparability and reuse of data. Environmental Research 245:118072.

Shaw, B. J., Ramsden, C. S., Turner, A., and Handy, R. D. (2013). A simplified method for determining titanium from TiO_2 nanoparticles in fish tissue with a concomitant multi-element analysis. Chemosphere, 92, 1136-1144.

Wang, F., Tharaud, M. and Benedetti, M.F. (2024). Advancing surface river water preparation for nanoparticle quantification and characterization using spICP-MS or spICP-ToF-MS. *Microchemical Journal*, *203*, p.110843.

Zhu, X., Wang, J., Zhang, X., Chang, Y., Chen, Y. (2010). Trophic transfer of TiO2 nanoparticles from daphnia to zebrafish in a simplified freshwater food chain. Chemosphere 79, 928–933.

Annex 1. Inter-Laboratory Comparison Study

The information in this Annex is intended to provide background on some of the guidance given in the main text (where this Annex is referred to). It provides further information on the inter-laboratory comparison study (ILC) that was performed in support of this Guidance Document. Within the ILC three aspects were investigated relevant for the reproducibility of the protocol: stock suspension preparation and characterization, feed spiking and testing for leaching from feed. After a general introduction on the setup of the ILC, each of the aspects are further detailed in methodology and results are shown. This annex is included only as an example of bioaccumulation studies of nanomaterials in fish, but the methodologies (e.g. dispersion protocol used) and considerations presented are not prescriptive.

Three laboratories from Universidad d'Aveiro (Portugal), University of Plymouth (UK) and National Institute for Agricultural and Food Research and Technology (INIA), CSIC (Spain) have participated in the ILC following the protocol described below. Each participating laboratory used their own instruments/equipment. Examples of equipment were provided in the methodology. However, deviations from these examples were allowed, provided that details of the specific equipment and labware used were reported. Probe sonication calibration according to the NANoREG-ECOTOX Probe Sonication Calibration SOP (Booth and Jensen, 2015a) was advised. The water used for leaching tests should mimic the aquarium water used for TG 305 (OECD 2012) tests and be at the appropriate temperature according to the test organism maintenance (e.g. for rainbow trout, fish bioaccumulation tests: 13-17 ºC). Feed could be selected by participating laboratories according to suitability, appropriateness for species and availability. Analysis of potential background concentrations of chemicals of interest and their levels in feed should be established prior to spiking to ensure study feasibility at relevant concentrations.

Four nanomaterials were distributed to each participant laboratory:

- CuO spherical: CuO nanomaterial from Merck (< 50 nm manufacturer reported TEM particle size, surface area of 29 m^2/g; CAS: 1317-38-0 Product number: 544868)
- CuO rod: Rod-shaped CuO nanomaterial from Merck (manufacturer reported diameter × length: 10-12 nm × 75-100 nm, surface area 60-100 m^2/g; CAS: 1317-38-0 Product number: 792004)
- CdTeQD-COOH: COOH coated CdTe quantum dots (QDs) from PlasmaChem GmbH (diameter of 3-5 nm according to the manufacturer; CAS: 1306-25-8; Lot YF140402)
- CdTeQD-PEG: PEG coated CdTe quantum dots from PlasmaChem GmbH (diameter of 3-5 nm) according to the manufacturer, CAS: 1306-25-8; Lot YF140402)

CdTeQDs were made specifically for the NANOSOLUTIONS EU project and have been extensively characterised (e.g., Vassallo et al., 2018).

For the final analysis of the generated samples, participating partners used their own methodology with careful instrument calibration and use of quality controls to ensure reliable total metal analysis.

The study design required the analysis of a total of 96 samples per nanomaterial.

Stock suspensions preparation and characterization

Aqueous suspensions of spherical and rod-shaped CuO nanomaterials, were prepared by applying the standard NANoREG-ECOTOX dispersion protocol (Booth and Jensen 2015b). Accordingly, samples were

prepared in ultrapure water only (Milli-Q water, 18.2 MΩ cm at 25 °C), but at concentrations of 350 mg Cu/L and 2500 mg Cu/L. For this, 4.2 mg and 30.03 mg of CuO nanomaterials are weighed in glass vials and dispersed in 10 mL of Milli-Q water to generate the low and high Cu concentrations mentioned, respectively. Dispersions were then sonicated using a probe sonicator and an ice water bath setup for an appropriate amount of time to deliver 7.35 ± 0.05 Watts of energy (see NANoREG-ECOTOX standard operation procedure for setup and calibration details). Three independent preparations for each concentration and nanomaterial were needed. Particle size distributions and Cu concentrations of each stock suspension needed to be determined (e.g., using DLS/NTA and ICP-MS/OES respectively). Due to the nature of the samples (e.g., susceptibility to sedimentation/precipitation), the protocol highlighted the importance of particle size distribution measurements and of taking samples for total metal analysis of the nanomaterial dispersions immediately after sonication. Samples could be stored at -20ºC until required for metal analysis but their stability should be studied.

Aqueous suspensions of COOH and PEG coated CdTe quantum dots, were prepared in ultrapure water (Milli-Q water, 18.2 MΩ cm at 25 °C) at stock concentrations of 50 and 500 mg QD/L. For this, 7.5 mg of sample was weighed and suspended in either 150 mL or 15 mL Milli-Q water in amber glass vials to generate low and high concentrations respectively, and the dispersions are shaken gently by hand, for 10 seconds (no sonication). Three independent preparations for each concentration and sample were needed. Particle size distributions and total Cd and Te concentrations of each stock suspension needed to be determined. Again, due to the nature of the samples (e.g. susceptibility to sedimentation/precipitation), the protocol highlighted the importance of particle size distribution measurements and of taking samples for metal analysis immediately after agitation.

Feed spiking

The procedure is illustrated in Figure 2 in Section 5 of this document. The stock suspensions prepared were used immediately after preparation/sonication to spike the fish feed. An aliquot of the suspension was directly added to the feed using a direct addition and mixing technique. A suspension volume to feed ratio of 0.2 mL/g feed was selected. Aliquots of 1 g of feed (sufficient for the analysis) were placed in glass beakers and 0.2 mL of stock suspension was directly added to the pellets using a pipette. Then, the suspension and pellets were mixed with a glass rod until the feed was homogeneously wetted. The suspension was added stepwise, with the first 0.1 mL and mixed, and then the second 0.1 mL in the same manner. Use of the prepared stock suspensions allowed a final spiked concentration of 70 and 500 mg Cu/kg feed for spherical and rod-shaped CuO nanomaterials respectively, and 10 and 100 mg CdTe QDs/kg feed for the COOH- and PEG-coated CdTe QDs. Feed was spiked from each three independently prepared stock suspension (stock 1, 2 and 3). Three independent spikings from each stock suspension were prepared in beakers (beaker 1, 2 and 3). From each of these beakers three spiked feed subsamples were analysed for metal content of the nanomaterial. Additionally, three replicates of control feed were spiked with Milli-Q water in the same way. Spiked feed was left to dry overnight at ambient room temperature (for QDs, also under dark conditions) and the next day transferred to an oven/incubator (< 30 ºC) to dry to a constant weight. The spiked diets were then stored under refrigeration (at 4 ºC) in a sealed airtight container (QDs also protected from light). The cool temperature is necessary to limit any fungal/bacterial growth in the feed, and storing feed at room temperature after spiking is to be avoided. These samples were analysed to quantify the total concentration of Cu or Cd and Te, as appropriate.

Leaching test

TG 305 (OECD 2012) warns (paragraph 142) about the fact that test substances can appear in the test medium as a result of leaching from food. Although the analysis in water or feed is not mandatory in TG it is advisable to perform leaching experiments and appropriate analysis for an accurate estimation of the

dose ingested by fish and possible additional exposure through the water. In the present experiment, leaching from feed was assessed (illustrated in Figure 2 in Section 5 of this document). For that, three glass beakers were used to add 100 mg of the spiked feed from each replicate beaker from one of the stock suspensions prepared. Then 10 mL of the aquarium water was added. Subsequently, the immersed feed was filtered by a filter paper and dried overnight at ambient temperature on the filter paper. If needed, these feed samples should be dried to constant weight (e.g. oven/incubator at < 30 ºC) before its preparation for analysis. These samples provided information of nanomaterial leaching at time 0. To study the leaching after 5 min immersion, another three beakers were prepared as indicated and then placed on an orbital shaker (100 rpm) for 5 min and then filtered. Also, a leaching test was done with control feed in the same way. These feed samples were analysed to quantify the total concentration of Cu or Cd and Te, as appropriate.

Results

Repeatability and recovery of the method within each laboratory and reproducibility between laboratories was assessed. The acceptable limits for an analytical method defined by the Association of Official Analytical Chemists (AOAC) International (2016) (AOAC, 2016) depends on the concentration of the analyte (Table 1). These limits are based on soluble chemical substances. Due to the nature of nanomaterials and their tendency to agglomerate in suspension, a higher relative standard deviation (RSD) for repeatability and reproducibility should be expected. In Table 1 we propose acceptable limits for nanomaterials.

Table 1. Expected repeatability, recovery and reproducibility of an analytical method as a function of analyte concentration (AOAC, 2016) and suggested values for nanomaterials.

Concentration	Repeatability[1]	Repeatability for nanomaterials	Recovery[2]	Reproducibility[3]	Reproducibility for nanomaterials
mg/kg or mg/L	RSD (%)[4]	RSD (%)[4]	(%)	RSD (%)	RSD (%)
1000	3.7	10	95-105	6	15
100	5.3	10	90-107	8	15
10	7.3	15	80-110	11	20
1	11	15	80-110	16	20
0.1	15	20	80-110	22	20

1. Repeatability: Conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
2. Recovery: Percentage of the analyte that is recovered when the test sample is analysed using the entire method.
3. Reproducibility: Conditions where independent test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.
4. RSD: relative standard deviation expressed in percentage.

Table 2 presents the results of the size distribution characterization of three independent stock suspensions. Laboratory 1 used a nano-tracking (NTA) device for the characterization whereas laboratories 2 and 3 used a dynamic light scattering (DLS) instrument. Triplicates of three stock suspensions were measured. The RSD for the repeatability of the hydrodynamic diameter mean peak (when NTA was used) or the Z-average (when DLS was used) of three independent stock suspensions are in most of the cases below 10 or 15 % (Table 2).

Table 3 shows the measured concentration of total Cu concentration for CuO spherical and CuO rod stock suspensions. Within laboratories, the repeatability was below 7% (except for one of the results) which is acceptable for the concentrations assessed of 350 and 2500 mg Cu/L. The reproducibility between laboratories was acceptable (RSD < 15%) with the exception of the stock suspensions of CuO spherical at the highest concentration of 2500 mg Cu/L. In this case, looking at the recoveries obtained by Laboratories 1 and 2 of the stock suspensions lower than 60% and higher than 120%, respectively, we

can conclude that something went wrong with the Cu measurements of this concentration. Apart from this exception, the recovery of the metal from both CuO nanomaterials was appropriate following the NANoREG-ECOTOX SOP (recoveries between 71 and 95%). Similar conclusions about repeatability and reproducibility were observed for CdTeQD-COOH and CdTeQD-PEG stock suspensions, especially for the measurements of Cd (Table 4). The RSD for repeatability for concentrations in the three stock suspensions of about 15 and 150 mg/L of Cd in both quantum dots was in the range of 1 and 7 %. The RSD for reproducibility of each stock suspension within laboratories for Cd was in the range of 1 to 16%. The recoveries of the Cd metal from the stock suspensions were about 30% for the three laboratories. Some difficulties were found for the measurements of Te in the QDs for Laboratory 2 and results are not reported in Table 4.

The direct addition protocol showed to be appropriate to obtain homogeneous and reproducible spiked feed within laboratories for the four nanomaterials. The RSD for the repeatability of the spiking process was in most of the cases below 10% within each of the laboratories for the total Cu metal measured from the CuO spherical and CuO rod nanomaterials and for total Cd measurements from both QDs. However, the reproducibility between the three laboratories was not so good with an RSD higher than 20% due to the lower recoveries of these metals obtained by Laboratory 2 (Table 5, Table 6, Table 7, and Table 9). Looking at the similar results of recoveries of these metals in Laboratories 1 and 3, we can conclude that something was wrong with the analysis for Laboratory 2. The variability observed within and between laboratories for the measurement of tellurium in the QDs (Table 8 and Table 10) is probably due to analytical difficulties with this metal.

The results of the leaching study of the nanomaterial from the feed to the aquarium water after 5 min immersion are reported in Table 11. Laboratories 1 and 3 reported recoveries of total Cu from both CuO nanomaterials ranging from 68 to 97% after 5 min immersion indicating low losses of the spiked nanomaterial. The RSD corresponding to the repeatability within laboratories was generally below 15 %. The recoveries in the two laboratories were very similar. The leaching study with both QDs was only conducted by Laboratory 3. After 5 min immersion the loss of total Cd and Te from the QDs was in most of the cases lower than 15%.

Taking all these results together, it can be concluded that the method proposed of a direct addition and mixing for spiking feed with a nanomaterial aqueous suspension provides good repeatability, recovery and reproducibility.

References

- AOAC International (2016). Association of Official Analytical Chemists (AOAC) Official methods of analysis. Appendix F: Guidelines for Standard Method Performance Requirements. Available online: https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf
- Booth, A. and Jensen,K.A. (2015a) NANoREG D4.12 SOP Probe Sonicator Calibration for ecotoxicological testing version 3. https://www.rivm.nl/sites/default/files/2018-11/NANoREG%20D4.12%20SOP%20Probe%20Sonicator%20Calibration%20for%20ecotoxicological%20testing.pdf
- Booth, A., Jensen, K.A. (2015b). SOP NANoREG-ECOTOXEcotox. Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media. Preparation and physicochemical characterisation of manufactured nanomaterials (MNMs) in environmental fate and ecotoxicity exposure media. Dispersion Standard Operating Procedure. Version 6. https://safenano.re.kr/download.do?SEQ=177
- OECD (2012), Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris, https://doi.org/10.1787/9789264185296-en.Vassallo, J., Besinis, A., Boden, R. and Handy, R.D.

(2018). The minimum inhibitory concentration (MIC) assay with Escherichia coli: An early tier in the environmental hazard assessment of nanomaterials?. Ecotoxicology and Environmental Safety, 162, pp.633-646.

Table 2. Size distribution characterization of stock suspensions prepared in triplicate per each nanomaterial by the three laboratories. Results from laboratory 1 are the mean of measurements of three aliquots of stock 1. Results of laboratories 2 and 3 are the mean of three means obtained from three aliquots of each stock suspension 1, 2 and 3.

	Lab.1 Stock1 by NTA[1]	Lab.2 Stock1.2.3 by DLS[2]	Lab.3 Stock1.2.3 by DLS[2]		Lab.1 Stock1 by NTA[1]	Lab.2 Stock1.2.3 by DLS[2]	Lab.3 Stock1.2.3 by DLS[2]
	Mean peak (d.nm)	Z-average (d.nm)	Z-average (d.nm)		Mean peak (d.nm)	Z-average (d.nm)	Z-average (d.nm)
CuO spherical				**CuO spherical**			
350 mg Cu/L				**2500 mg Cu/L**			
mean	131.3	298.6	793.7*	mean	158.7	349.8	320.5*
SD	2.1	26.3	60.3	SD	32.7	84.3	44.2
RSD	1.6	8.8	7.6	RSD	20.6	24.1	13.8
CuO rod				**CuO rod**			
350 mg Cu/L				**2500 mg Cu/L**			
mean	148.0	2864.8		mean	163.3	1146.7	
SD	89.1	406.7		SD	11.0	12.2	
RSD	60.2	14.2		RSD	6.7	1.1	
CdTeQD-COOH				**CdTeQD-COOH**			
50 mg QD/L				**500 mg QD/L**			
mean	206.3	233.0	193.7	mean	206.7	225.5	180.4
SD	11.5	2.4	10.3	SD	3.8	1.8	0.7
RSD	5.6	1.0	5.3	RSD	1.8	0.8	0.4
CdTeQD-PEG				**CdTeQD-PEG**			
50 mg QD/L				**500 mg QD/L**			
mean	168.3	307.3	306.4	mean	176.7	228.3	309.1
SD	34.5	15.9	17.3	SD	21.6	7.9	94.3
RSD	20.5	5.2	5.7	RSD	12.2	3.4	30.5

*mean triplicate stock 1

1. NTA, Nano Tracking Analysis

2. DLS, Dynamic Light Scattering

Table 3. Measured total Cu concentration (mg/L) of CuO spherical and CuO rod stock suspensions. Results are expressed as mean±SD, n=3 aliquots

CuO spherical									
stock suspension	**Stock 1**		**Stock 2**		**Stock 3**	***Nominal****			
350 mg Cu/ L	***304***		***359***		***367***	***Lab.1***			
	350		***350***		***350***	***Lab.2***			
	250					***Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
	mean	SD	mean	SD	mean	SD			
Lab.1	242	41	288	7	295	8			
Lab.2	284	37	266	4	263	33			
Lab.3	215	7							
mean	247		277		279				
SD	35		15		22				
RSD (%)	14		5		8		mean	SD	RSD
Lab.1 recovery	80		80		80		80	0	0
Lab.2 recovery	81		76		75		77	3	4
Lab.3 recovery	86						86		

CuO spherical									
stock suspension	**Stock 1**		**Stock 2**		**Stock 3**	***Nominal****			
2500 mg Cu / L	***2484***		***2429***		***2484***	***Lab.1***			
	2500		***2500***		***2500***	***Lab.2***			
	2500					***Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
	mean	SD	mean	SD	mean	SD			
Lab.1	1544	68	1422	81	1154	268			
Lab.2	3453	225	3878	306	3906	331			
Lab.3	2400	100							
mean	2466		2650		2530				
SD	956		1736		1946				
RSD (%)	39		66		77		mean	SD	RSD
Lab.1 recovery	62		59		46		56	8	15
Lab.2 recovery	138		155		156		150	10	7
Lab.3 recovery	96						96		

CuO rod									
stock suspension	**Stock 1**		**Stock 2**		**Stock 3**	***Nominal****			
350 mg Cu/ L	***367***		***344***		***320***	***Lab.1***			
	350		***350***		***350***	***Lab.2***			
	336					***Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
	mean	SD	mean	SD	mean	SD			
Lab.1	270	11	278	8	228	7			
Lab.2	326	16	312	31	309	5			
Lab.3	290	2							
mean	295		295		268				
SD	28		25		58				
RSD (%)	9		8		22		mean	SD	RSD
Lab.1 recovery	74		81		71		75	5	7

CuO rod									
stock suspension	**Stock 1**		**Stock 2**		**Stock 3**	***Nominal****			
2500 mg Cu / L	***2445***		***2397***		***2397***	***Lab.1***			
	2500		***2500***		***2500***	***Lab.2***			
	2397					***Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
	mean	SD	mean	SD	mean	SD			
Lab.1	1990	31	1874	59	1988	14			
Lab.2	2331	133	2328	58	2368	110			
Lab.3	2008	21							
mean	2110		2101		2178				
SD	192		321		269				
RSD (%)	9		15		12		mean	SD	RSD
Lab.1 recovery	81		78		83		81	2	3

	Stock 1	Stock 2	Stock 3	Mean	SD	RSD		Stock 1	Stock 2	Stock 3	Mean	SD	RSD
Lab.2 recovery	93	89	88	90	2	3	Lab.2 recovery	93	93	95	94	1	1
Lab.3 recovery	86			86			Lab.3 recovery	84			84		

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 4. Measured total Cd (mg/L) and Te (mg/L) concentrations of CdTeQD-COOH and CdTeQD-PEG stock suspensions. Results are expressed as mean±SD, n=3 aliquots

CdTeQD-COOH/ L (50 mg / L)	Stock 1		Stock 2		Stock 3				
Cadmium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	15	0	14	0	15	0			
Lab.2	15	0	14	0	14	1			
Lab.3	13	0	14	0	14	0			
mean	14		14		14				
SD	1		0		0				
RSD (%)	8		3		3		Mean	SD	RSD
Lab.1 recovery	31		29		29		30	1	3
Lab.2 recovery	29		27		28		28	1	4
Lab.3 recovery	26		27		28		27	1	3
Tellurium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	3	0	3	0	3	0			
Lab.2									
Lab.3	2	0	2	0	2	0			
mean	3		3		3				
SD	1		1		1				
RSD (%)	42		36		33		Mean	SD	RSD
Lab.1 recovery	7		7		7		7	0	2
Lab.2 recovery									
Lab.3 recovery	4		4		4		4	0	5
CdTeQD-PEG/ L (50 mg / L)	**Stock 1**		**Stock 2**		**Stock 3**				
Cadmium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	16	0	17	0	15	0			
Lab.2	16	0	16	0	15	0			
Lab.3	16	0	14	0	14	0			

CdTeQD-COOH/ L (500 mg / L)	Stock 1		Stock 2		Stock 3				
Cadmium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	146	2	149	1	146	1			
Lab.2	145	6	150	1	160	5			
Lab.3	137	0	135	1	137	1			
mean	142		144		147				
SD	5		8		12				
RSD (%)	4		6		8		Mean	SD	RSD
Lab.1 recovery	29		30		29		29	0	1
Lab.2 recovery	29		30		32		30	1	5
Lab.3 recovery	27		27		27		27	0	1
Tellurium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	14	10	8	1	8	1			
Lab.2									
Lab.3	19	1	19	1	19	1			
mean	16		13		13				
SD	3		8		7				
RSD (%)	20		59		57		Mean	SD	RSD
Lab.1 recovery	3		2		2		2	1	36
Lab.2 recovery									
Lab.3 recovery	4		4		4		4	0	1
CdTeQD-PEG/ L (500 mg / L)	**Stock 1**		**Stock 2**		**Stock 3**				
Cadmium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	150	1	157	2	156	5			
Lab.2	197	13	177	4	189	8			
Lab.3	154	0	154	1	149	0			

mean	16		16		15				
SD	0		1		1				
RSD (%)	1		8		6		Mean	SD	RSD
Lab.1 recovery	31		34		31		32	2	5
Lab.2 recovery	32		32		31		31	0	1
Lab.3 recovery	32		29		28		29	2	7
Tellurium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	4	0	4	0	4	0			
Lab.2									
Lab.3	2	0	2	0	2	0			
mean	3		3		3				
SD	1		1		1				
RSD (%)	31		33		46		Mean	SD	RSD
Lab.1 recovery	8		8		8		8	0	1
Lab.2 recovery									
Lab.3 recovery	5		5		4		5	1	12

mean	167		162		165				
SD	26		12		21				
RSD (%)	16		8		13		Mean	SD	RSD
Lab.1 recovery	30		31		31		31	1	2
Lab.2 recovery	39		35		38		37	2	5
Lab.3 recovery	31		31		30		30	1	2
Tellurium conc.	mean	SD	mean	SD	mean	SD			
Lab.1	11	1	18	12	12	1			
Lab.2									
Lab.3	23	0	24	0	23	0			
mean	17		21		18				
SD	9		5		8				
RSD (%)	49		22		43		Mean	SD	RSD
Lab.1 recovery	2		4		2		3	1	25
Lab.2 recovery									
Lab.3 recovery	5		5		5		5	0	3

SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 5. Measured total Cu concentration (mg/kg) of CuO spherical in spiked feed. Results are expressed as mean±SD, n=3 aliquots

	60		*72*		*73*	*nominal Lab.1**			
Spiked feed	*57*		*53*		*53*	*nominal Lab.2**			
70 mg/kg	*70*		*70*		*70*	*nominal Lab.3**			
	Stock 1		**Stock 2**		**Stock 3**				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	43	1	58	1	53	3			
Beaker 2	65	27	55	8	58	3			
Beaker 3	53	1	58	3	55	2			
Recovery									
Beaker 1	71		81		74				
Beaker 2	108		77		80				
Beaker 3	88		82		76		**mean**	**SD**	**RSD**
mean	**89**		**80**		**76**		**82**	**7**	**8**
SD	**18**		**2**		**3**				
RSD (%)	**21**		**3**		**4**				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	16	2	19	2	20	1			
Beaker 2	17	2	18	1	15	1			
Beaker 3	20	1	22	2	20	1			
Recovery									
Beaker 1	28		37		39				
Beaker 2	30		33		29				
Beaker 3	36		42		38		**mean**	**SD**	**RSD**
mean	**31**		**37**		**35**		**35**	**3**	**9**
SD	**4**		**5**		**6**				
RSD (%)	**12**		**12**		**16**				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	70	8	36	3	44	3			
Recovery							**mean**	**SD**	**RSD**
Beaker 1	100		52		63		**72**	**25**	**35**

	495		*484*		*492*	*nominal Lab.1**			
Spiked feed	*691*		*776*		*781*	*nominal Lab.2**			
500 mg/kg	*500*		*500*		*500*	*nominal Lab.3**			
	Stock 1		**Stock 2**		**Stock 3**				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	335	9	306	12	311	9			
Beaker 2	304	7	350	7	286	5			
Beaker 3	316	14	344	13	307	4			
Recovery									
Beaker 1	68		63		63				
Beaker 2	61		72		58				
Beaker 3	64		71		62		**mean**	**SD**	**RSD**
mean	**64**		**69**		**61**		**65**	**4**	**6**
SD	**3**		**5**		**3**				
RSD (%)	**5**		**7**		**4**				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	182	11	152	9	151	8			
Beaker 2	177	13	153	7	147	2			
Beaker 3	176	8	143	4	139	7			
Recovery									
Beaker 1	26		20		19				
Beaker 2	26		20		19				
Beaker 3	25		18		18		**mean**	**SD**	**RSD**
mean	**26**		**19**		**19**		**21**	**4**	**19**
SD	**0**		**1**		**1**				
RSD (%)	**2**		**4**		**4**				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	399	33	318	30	344	21			
Recovery							**mean**	**SD**	**RSD**
Beaker 1	80		64		69		**71**	**8**	**12**

Lab 1, 2, 3	
mean	**63**
SD	**25**
RSD (%)	**40**

Lab 1, 2, 3	
mean	**52**
SD	**27**
RSD (%)	**52**

Control feed	Rep1	Rep 2	Rep 3	mean	SD
Lab.1				2	1
Lab.2				8	0
Lab.3				11	4

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 6. Measured total Cu concentration (mg/Kg) of CuO rod in spiked feed. Results are expressed as mean±SD, n=3 aliquots

Spiked feed	**73**		**68**		**63**	**nominal Lab.1***			
70 mg/kg	**65**		**62**		**62**	**nominal Lab.2***			
	70		**70**		**70**	**nominal Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	43	2	55	3	44	1			
Beaker 2	50	9	57	3	49	3			
Beaker 3	53	3	49	5	46	0			
Recovery									
Beaker 1	58		81		70				
Beaker 2	69		83		78				
Beaker 3	73		71		73		mean	SD	RSD
mean	67		78		74		73	6	8
SD	8		6		4				
RSD (%)	11		8		5				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	22	2	20	1	22	1			
Beaker 2	22	0	21	0	18	1			
Beaker 3	19	6	21	2	20	1			
Recovery									
Beaker 1	33		32		36				
Beaker 2	33		33		29				
Beaker 3	29		34		32		mean	SD	RSD
mean	32		33		33		33	1	2
SD	2		1		3				
RSD (%)	7		3		10				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	58	4	55	3	56	2			
Recovery							mean	SD	RSD
Beaker 1	83		78		80		80	2	3

Spiked feed	**482**		**476**		**474**	**nominal Lab.1***			
500 mg/kg	**466**		**466**		**474**	**nominal Lab.2***			
	500		**500**		**500**	**nominal Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	361	26	331	32	312	27			
Beaker 2	371	34	343	17	365	17			
Beaker 3	379	21	332	25	363	18			
Recovery									
Beaker 1	75		70		66				
Beaker 2	77		72		77				
Beaker 3	79		70		77		mean	SD	RSD
mean	77		70		73		73	3	4
SD	2		1		6				
RSD (%)	2		2		9				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	169	8	166	1	157	4			
Beaker 2	176	5	160	5	161	8			
Beaker 3	188	5	171	2	169	6			
Recovery									
Beaker 1	36		36		33				
Beaker 2	38		34		34				
Beaker 3	40		37		36		mean	SD	RSD
mean	38		36		34		36	2	5
SD	2		1		1				
RSD (%)	5		3		4				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	571	15	396	7	398	22			
Recovery							mean	SD	RSD
Beaker 1	114		79		80		91	20	22

Lab. 1, 2, 3	
mean	62
SD	26
RSD (%)	42

Lab. 1, 2, 3	
mean	67
SD	28
RSD (%)	42

Control feed	Rep 1	Rep 2	Rep 3	mean	SD
Lab.1				2	1
Lab.2				9	1
Lab.3				10	1

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 7. Measured total Cd concentration (mg/Kg) of CdTeQD-COOH in spiked feed. Results are expressed as mean±SD, n=3 aliquots

Spiked feed	**3**		**3**		**3**		**nominal Lab.1***		
10 mg/kg	**3**		**3**		**3**		**nominal Lab.2***		
(~2,8 mg Cd/kg)	**3**		**3**		**3**		**nominal Lab.3***		
	Stock 1		**Stock 2**		**Stock 3**				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	3	0	3	0	3	0			
Beaker 2	3	0	3	0	3	0			
Beaker 3	3	0	3	0	3	0			
Recovery									
Beaker 1	107		97		111				
Beaker 2	109		100		113				
Beaker 3	99		111		108		Mean	SD	RSD
mean	105		103		111		106	4	4
SD	5		7		2				
RSD (%)	5		7		2				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	1	0	1	0	1	0			
Beaker 2	1	0	1	0	1	0			
Beaker 3	1	0	1	0	1	0			
Recovery									
Beaker 1	48		44		35				
Beaker 2	46		45		41				
Beaker 3	48		40		43		Mean	SD	RSD
mean	47		43		40		43	4	9
SD	1		3		4				
RSD (%)	3		6		10				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	3	0	3	0	2	0			
Beaker 2									
Beaker 3									
Recovery							Mean	SD	RSD

Spiked feed	**29**		**30**		**29**	**nominal Lab.1***			
100 mg/kg	**29**		**30**		**32**	**nominal Lab.2***			
(~28 mg Cd/kg)	**27**		**27**		**27**	**nominal Lab.3***			
	Stock 1		**Stock 2**		**Stock 3**				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	25	4	28	0	28	1			
Beaker 2	25	2	28	2	30	2			
Beaker 3	28	2	28	0	30	0			
Recovery									
Beaker 1	85		96		94				
Beaker 2	87		94		102				
Beaker 3	97		96		104		Mean	SD	RSD
mean	90		95		100		95	5	6
SD	7		1		5				
RSD (%)	8		1		5				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	13	0	12	1	13	0			
Beaker 2	13	1	13	0	13	0			
Beaker 3	13	0	13	0	11	2			
Recovery									
Beaker 1	44		41		40				
Beaker 2	43		43		41				
Beaker 3	45		42		34		Mean	SD	RSD
mean	44		42		38		42	3	7
SD	1		1		4				
RSD (%)	2		3		10				
	Stock 1		**Stock 2**		**Stock 3**				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	27	1	28	1	24	1			
Beaker 2									
Beaker 3									
Recovery							Mean	SD	RSD

Beaker 1	101	95	86	94	7	8	Beaker 1	98	104	89	97	8	8
Beaker 2							Beaker 2						
Beaker 3							Beaker 3						
Lab. 1, 2, 3							**Lab. 1, 2, 3**						
mean	81						mean	78					
SD	33						SD	32					
RSD (%)	41						RSD (%)	40					
Control feed	Stock 1	Rep 2	Rep 3	Mean(Cd)	SD								
Lab.1	0	0	0	0	0								
Lab.2	0	0	0	0	0		Lab.3				0	0	

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 8. Measured total Te (mg/Kg) concentration of CdTeQD-COOH in spiked feed. Results are expressed as mean±SD, n=3 aliquots

Spiked feed	1		1		1				
10 mg/kg	0		0		0				
(~0,4 mg Te/kg)	0		0		0				
	Stock 1		Stock 2		Stock 3				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	1	0	0	0	1	0			
Beaker 2	1	0	0	0	1	0			
Beaker 3	0	0	1	0	1	0			
Recovery									
Beaker 1	78		72		78				
Beaker 2	76		72		77				
Beaker 3	71		76		77		Mean	SD	RSD
mean	75		73		77		75	2	3
SD	4		3		1				
RSD (%)	5		3		1				
	Stock 1		Stock 2		Stock 3				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	0	0	0	0	0	0			
Beaker 2	0	0	0	0	0	0			
Beaker 3	0	0	0	0	0	0			
Recovery									
Beaker 1	90		55		8				
Beaker 2	115		83		35				
Beaker 3	43		75		93		Mean	SD	RSD
mean	83		71		45		66	19	29
SD	37		14		43				
RSD (%)	45		20		96				
	Stock 1		Stock 2		Stock 3				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	0	0	0	0	0	0			
Beaker 2									
Beaker 3									
Recovery							Mean	SD	RSD

Spiked feed	3		2		2				
100 mg/kg	4		4		4				
(~4 mg Te/kg)	4		4		4				
	Stock 1		Stock 2		Stock 3				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	4	1	4	0	4	0			
Beaker 2	4	0	4	0	4	0			
Beaker 3	4	0	4	0	4	0			
Recovery									
Beaker 1	136		286		265				
Beaker 2	144		281		280				
Beaker 3	158		286		284		Mean	SD	RSD
mean	146		285		276		236	78	33
SD	11		3		10				
RSD (%)	7		1		4				
	Stock 1		Stock 2		Stock 3				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	3	0	2	1	2	0			
Beaker 2	3	0	2	1	2	0			
Beaker 3	3	0	2	0	2	0			
Recovery									
Beaker 1	69		46		60				
Beaker 2	63		52		60				
Beaker 3	64		59		50		Mean	SD	RSD
mean	65		52		57		58	7	11
SD	3		7		6				
RSD (%)	5		13		10				
	Stock 1		Stock 2		Stock 3				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	4	0	4	0	3	0			
Beaker 2									
Beaker 3									
Recovery							Mean	SD	RSD

Beaker 1	97		87		79		88	9	11	Beaker 1	95	99	88	94	6	6
Beaker 2										Beaker 2						
Beaker 3										Beaker 3						
Lab. 1, 2, 3										**Lab. 1, 2, 3**						
mean	76									mean	129					
SD	11									SD	94					
RSD (%)	14									RSD (%)	73					
Control feed	Stock 1	SD	Rep 2	SD	Rep 3	SD	Mean(Te)	SD								
Lab.1	0		0		0		0		Lab.3				<LOQ			
Lab.2							0						LOQ=0,5 µg/L			

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 9. Measured total Cd concentration of CdTeQD-PEG in spiked feed. Results are expressed as mean±SD, n=3 aliquots

	3		*3*		*3*		*nominal Lab.1**			
Spiked feed	*3*		*3*		*3*		*nominal Lab.2**			
10 mg/kg (~3 mg Cd/kg)	*3*		*3*		*3*		*nominal Lab.3**			
	Stock 1		***Stock 2***		***Stock 3***					
Lab.1	mean	SD	mean	SD	mean	SD				
Beaker 1	3	0	3	0	3	0				
Beaker 2	3	0	3	0	3	0				
Beaker 3	3	0	3	0	3	1				
Recovery										
Beaker 1	98		87		111					
Beaker 2	93		89		102					
Beaker 3	106		86		111		**Mean**	**SD**	**RSD**	
mean	**99**		**87**		**108**		**98**	**10**	**11**	
SD	**6**		**1**		**5**					
RSD (%)	**6**		**2**		**5**					
	Stock 1		***Stock 2***		***Stock 3***					
Lab.2	mean	SD	mean	SD	mean	SD				
Beaker 1	2	0	1	0	2	0				
Beaker 2	1	0	1	0	2	0				
Beaker 3	2	0	1	0	1	0				
Recovery										
Beaker 1	47		41		50					
Beaker 2	47		34		50					
Beaker 3	49		37		47		**Mean**	**SD**	**RSD**	
mean	**48**		**37**		**49**		**45**	**7**	**15**	
SD	**1**		**3**		**2**					
RSD (%)	**2**		**9**		**3**					
	Stock 1		***Stock 2***		***Stock 3***					
Lab.3	mean	SD	mean	SD	mean	SD				
Beaker 1	3	0	3	0	3	0				
Recovery							**Mean**	**SD**	**RSD**	

	30		*31*		*31*		*nominal Lab.1**			
Spiked feed	*39*		*35*		*38*		*nominal Lab.2**			
100 mg/kg (~30 mg Cd/kg)	*31*		*31*		*30*		*nominal Lab.3**			
	Stock 1		***Stock 2***		***Stock 3***					
Lab.1	mean	SD	mean	SD	mean	SD				
Beaker 1	32	2	32	1	26	3				
Beaker 2	29	1	31	7	27	0				
Beaker 3	31	2	29	1	30	3				
Recovery										
Beaker 1	108		101		82					
Beaker 2	97		98		86					
Beaker 3	105		93		97			**Mean**	**SD**	**RSD**
mean	**103**		**97**		**88**			**96**	**8**	**8**
SD	**6**		**4**		**8**					
RSD (%)	**6**		**4**		**9**					
	Stock 1		***Stock 2***		***Stock 3***					
Lab.2	mean	SD	mean	SD	mean	SD				
Beaker 1	16	1	16	1	16	2				
Beaker 2	17	1	16	1	15	1				
Beaker 3	16	0	16	1	16	1				
Recovery										
Beaker 1	42		45		42					
Beaker 2	43		45		41					
Beaker 3	41		46		41			**Mean**	**SD**	**RSD**
mean	**42**		**45**		**41**			**43**	**2**	**5**
SD	**1**		**1**		**0**					
RSD (%)	**2**		**1**		**1**					
	Stock 1		***Stock 2***		***Stock 3***					
Lab.3	mean	SD	mean	SD	mean	SD				
Beaker 1	26	1	26	1	26	0				
Recovery								**Mean**	**SD**	**RSD**

Beaker 1	98	94	101	**98**	**4**	**4**	Beaker 1	86	83	87	**85**	**2**	**2**

Lab. 1, 2, 3	
mean	**80**
SD	**31**
RSD (%)	**38**

Lab. 1, 2, 3	
mean	**75**
SD	**28**
RSD (%)	**38**

Control feed	Rep 1	Rep 2	Rep 3	**Mean(Cd)**	**SD**
Lab.1	0	0	0	0	0
Lab.2	0	0	0	0	0
Lab.3				0	0

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 10. Measured total Te concentration of CdTeQD-PEG in spiked feed. Results are expressed as mean±SD, n=3 aliquots

	1		*1*		*1*				
Spiked feed Rep.1	*1*		*1*		*1*				
10 mg/kg (~0,5 mg Te/kg)	*0*		*0*		*0*				
	Stock 1		*Stock 2*		*Stock 3*				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	1	0	1	0	1	0			
Beaker 2	1	0	1	0	1	0			
Beaker 3	1	0	1	0	1	0			
Recovery									
Beaker 1	79		82		71				
Beaker 2	83		84		78				
Beaker 3	76		80		78		**Mean(Te)**	**SD**	**RSD**
mean	**79**		**82**		**76**		**79**	**3**	**4**
SD	**3**		**2**		**4**				
RSD (%)	**4**		**2**		**6**				
	Stock 1		*Stock 2*		*Stock 3*				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	0	0	1	0	1	0			
Beaker 2	1	0	1	0	1	0			
Beaker 3	1	0	1	0	1	0			
Recovery									
Beaker 1	46		138		136				
Beaker 2	154		140		156				
Beaker 3	136		144		104		**Mean(Te)**	**SD**	**RSD**
mean	**112**		**141**		**132**		**128**	**15**	**11**
SD	**58**		**3**		**26**				
RSD (%)	**52**		**2**		**20**				
	Stock 1		*Stock 2*		*Stock 3*				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	0	0	0	0	1	0			
Recovery							**Mean(Te)**	**SD**	**RSD**
Beaker 1	94		95		126		**105**	**18**	**17**

	2		*4*		*2*				
Spiked feed	*4*		*4*		*4*				
100 mg/kg (4 mg Te/kg)	*5*		*5*		*5*				
	Stock 1		*Stock 2*		*Stock 3*				
Lab.1	mean	SD	mean	SD	mean	SD			
Beaker 1	5	0	3	0	3	0			
Beaker 2	5	0	3	0	3	0			
Beaker 3	5	0	3	0	3	1			
Recovery									
Beaker 1	224		83		139				
Beaker 2	215		84		128				
Beaker 3	218		81		139		**Mean(Te)**	**SD**	**RSD**
mean	**219**		**83**		**135**		**146**	**69**	**47**
SD	**5**		**1**		**6**				
RSD (%)	**2**		**2**		**5**				
	Stock 1		*Stock 2*		*Stock 3*				
Lab.2	mean	SD	mean	SD	mean	SD			
Beaker 1	4	0	4	0	4	1			
Beaker 2	4	0	4	1	3	1			
Beaker 3	4	0	4	0	3	1			
Recovery									
Beaker 1	102		92		91				
Beaker 2	102		91		80				
Beaker 3	93		99		73		**Mean(Te)**	**SD**	**RSD**
mean	**99**		**94**		**81**		**91**	**9**	**10**
SD	**5**		**5**		**9**				
RSD (%)	**5**		**5**		**11**				
	Stock 1		*Stock 2*		*Stock 3*				
Lab.3	mean	SD	mean	SD	mean	SD			
Beaker 1	4	0	4	0	4	0			
Recovery							**Mean(Te)**	**SD**	**RSD**
Beaker 1	82		90		96		**89**	**7**	**8**

Lab. 1, 2, 3	
mean	**104**
SD	**8**
RSD (%)	**7**

Lab. 1, 2, 3	
mean	**109**
SD	**32**
RSD (%)	**29**

Control feed	Rep 1	Rep 2	Rep 3	Mean(Te)	SD
Lab.1	0	0	0	0	0
Lab.2				0	
Lab.3				<LOQ	
				LOQ=0,5 µg/L	

* Expected nominal concentration for the stock suspensions. SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.

Table 11. Leaching of nanomaterials from feed to aquarium water after 5 min immersion. Measurement of total metal of the nanomaterials in feed samples (mg/kg).

CuO spherical													
Spiked feed							***Spiked feed***						
70 mg/kg	Beak. 1	Beak. 2	Beak . 3				***500 mg/kg***	Beak . 1	Beak . 2	Beak . 3			
Before immersion				mean	SD	RSD	**Before immersion**				mean	SD	RSD
Lab.1	43	65	53	53	11	21	Lab.1	335	304	316	318	15	5
Lab.3				31	7		Lab.3				229	59	
After imm.							**After imm.**						
Lab.1	33	46	39	39	7	17	Lab.1	243	222	214	226	15	7
Lab.3	28	26	22	25	3	13	Lab.3	227	236	206	223	15	7
Recovery							**Recovery**						
Lab.1	78	72	73	74	3	5	Lab.1	73	73	68	71	3	4
Lab.3	92	83	70	82	11	13	Lab.3	99	103	90	97	7	7

CuO rod													
Spiked feed							***Spiked feed***						
70 mg/kg	Beak. 1	Beak. 2	Beak . 3				***500 mg/kg***	Beak . 1	Beak . 2	Beak . 3			
Before immersion				mean	SD	RSD	*Before immersion*				mean	SD	RSD
Lab.1	43	50	53	49	6	11	Lab.1	361	371	379	370	9	2
Lab.3	64	71	71	68	4	6	Lab.3	598	580	567	582	15	3
After imm.							After imm.						
Lab.1	34	39	24	32	8	24	Lab.1	326	278	261	288	34	12
Lab.3	63	62	55	60	5	8	Lab.3	509	501	535	515	18	3
Recovery							Recovery						
Lab.1	81	78	45	68	20	30	Lab.1	90	75	69	78	11	14
Lab.3	99	97	86	94	7	8	Lab.3	85	84	89	86	3	3

CdTe-COOH (Cadmium measurements)					
Spiked feed	Beak. 1	**Lab.3**	**Spiked feed**	Beak. 1	**Lab.3**
10 mg/kg	mean	SD	**100 mg/kg**	mean	SD
Before immersion	3	0	**Before immersion**	27	1
After imm	3	0	**After imm**	25	1
Recovery	91		**Recovery**	92	

CdTe-COOH (Tellurium measurements)					
Spiked feed	Beak. 1	**Lab.3**	**Spiked feed**	Beak. 1	**Lab.3**
10 mg/kg	mean	SD	**100 mg/kg**	mean	SD
Before immersion	0	0	**Before immersion**	4	0
After imm	0	0	**After imm**	3	0
Recovery	83		**Recovery**	90	

CdTe-PEG (Cadmium measurements)					
Spiked feed	Beak. 1	**Lab.3**	**Spiked feed**	Beak. 1	**Lab.3**

10 mg/kg	mean	SD	100 mg/kg	mean	SD
Before immersion	4	0	Before immersion	27	1
After imm	3	0	After imm	26	1
Recovery	91		Recovery	97	
		CdTe-PEG (Tellurium measurements)			
Spiked feed	Beak. 1	Lab.3	Spiked feed	Beak. 1	Lab.3
10 mg/kg	mean	SD	100 mg/kg	mean	SD
Before immersion	0	0	Before immersion	4	0
After imm	0	0	After imm	4	0
Recovery	79		Recovery	92	

SD, Standard Deviation; RSD, Relative Standard Deviation. Recovery, expressed as %.