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Final report

Standardization of Methods on the Fate and Behavior of Nanomaterials in Environmental Media – Dissolution and Dissolution Rate

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Abstract: Standardization of Methods on the Fate and Behavior of Nanomaterials in Environmental Media – Dissolution and Dissolution Rate

The objective of this project was to develop methods for determining the solubility and dissolution rate of nanomaterials (NMs) under conditions representative of the aquatic environment. These methods were designed to be cost-effective and reproducible, with the goal of establishing a universally applicable guideline within the framework of the OECD Test Guidelines program. Both, static batch procedures and dynamic flow-through procedures were developed. A ring test was conducted to evaluate the reproducibility of these methods.

The procedures are based on experiments in which the pH of the medium is passively maintained at a constant level using biochemical buffers (MES and MOPS). Separation of solid and dissolved fractions is achieved through ultrafiltration membranes, either in centrifugal ultrafiltration units (batch) or in filter holders (flow-through). The methods enable the determination of 24-hour solubility at pH 7, as well as 96-hour solubility at pH levels of 5, 7, and 8. Additionally, they allow for the determination of dissolution rates for moderately to poorly soluble NMs (with solubility approximately < 5 mg/L).

Kurzbeschreibung: Standardisierung von Methoden zum Verbleib und Verhalten von Nanomaterialien in Umweltmedien - Auflösung und Löslichkeitsrate

Ziel dieses Vorhabens war die Entwicklung von Verfahren zur Bestimmung der Löslichkeit und Auflösungsrate von Nanomaterialien (NM) unter Bedingungen der aquatischen Umwelt. Diese Verfahren sollten kosteneffizient und reproduzierbar sein, so dass aus ihnen eine allgemeingültige Richtlinie im Rahmen des OECD Testrichtlinienprogramms entstehen kann. Entwickelt wurden sowohl statische Batch-Verfahren als auch dynamische Durchflussverfahren. Im Rahmen eines Ringtests wurden die Verfahren auf ihre Reproduzierbarkeit überprüft.

Die Verfahren basieren auf Versuchen in denen der pH-Wert des Mediums mit Hilfe von biochemischen Puffern (MES und MOPS) passiv konstant gehalten wird. Die Separation von Feststoff- und gelösten Anteilen wird mit Hilfe von Ultrafiltrationsmembranen erreicht, entweder in Zentrifugal-Ultrafiltrationseinheiten (Batch) oder in Filterhaltern (Durchfluss). Die Verfahren ermöglichen die Bestimmung der 24-Stunden Löslichkeit bei pH 7 sowie die 96-Stunden Löslichkeit bei pH 5, 7 und 8. Darüber hinaus erlauben sie die Bestimmung der Auflösungsrate bei mittel bis wenig löslichen NMs (bei ca. < 5 mg/L Löslichkeit).

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List of abbreviations

Abbreviation	Explanation
GD	Guidance document
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
ILC	Inter-laboratory comparison
JEG	Joint WPMN-WNT expert group on environmental fate and ecotoxicity testing
MEPBS	4-[4-(2-Hydroxyethyl)piperazin-1-yl]butane-1-sulfonic acid
MES	2-morpholin-4-ylethanesulfonic acid
MOPS	3-(Morpholin-4-yl)propane-1-sulfonic acid
MWCO	Molecular weight cut-off (usually given in kilodaltons (kDa)
NM; NMs	Nanomaterial; Nanomaterials
NP; NPs	Nanoparticle; nanoparticles
OECD	Organisation for Economic Co-operation and Development
PTFE	Polytetrafluorethylene (Teflon)
SOP	Standard operating procedure
TG	Test Guideline
WNT	Working Group of National Co-ordinators of the TGs programme
WPMN	Working Party of Manufactured Nanomaterials

Summary

The aim of the project was to develop a methodological basis for establishing an OECD test guideline to assess the solubility and dissolution rate of nanomaterials (NMs). This encompassed not only the experimental approach but also the relevant composition of test media in which the dissolution of NMs would be assessed, the timeframe, sampling frequency, and methods for separating undissolved NMs from their dissolved components. Development was to refer to existing and developing test guidelines. The starting point was OECD Test Guideline TG 105 for *Water Solubility*, as well as OECD GD 29 on *Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media*.

To achieve this goal, both static (batch test) and dynamic (flow-through) methods were evaluated for their suitability. In the batch test, centrifugal ultrafiltration units with a 6 mL sample volume and a 10,000 g/mol (Dalton) molecular weight cutoff (MWCO) were selected to separate undissolved NM from the dissolved fraction. For the flow-through test, regenerated cellulose ultrafiltration membranes with a 45 mm diameter and 10,000 g/mol MWCO were chosen.

The pH value was identified as the most critical parameter that must be controlled and ideally kept constant during the test, as many substances exhibit pH-dependent solubility and dissolution rates. At the same time, the dissolution of substances in an unbuffered medium influences the pH, often by consuming protons (or releasing hydroxide ions). This effect must be prevented either actively (using an auto-titrator) or passively (by adding suitable buffers). Additionally, components in the medium should neither complex metal ions nor form poorly soluble compounds, as this would otherwise distort the results. Similarly, initiation or catalysis of redox reactions should be avoided. For reasons discussed in the report, active pH control was not used. The buffers used for pH stabilization are well selected *Good's buffers*, developed in biochemistry and known for their low metal complexation. MES buffer was chosen for a pH range of 5, and MOPS buffer for a pH range of 7 to 8. The composition of the media excluded substances found in natural waters that could form poorly soluble compounds. Regardless of the medium composition selected, the developed tests can be conducted with other media if they are compatible with ultrafiltration, for instance, and the pH is controlled or at least measured.

The methods developed in the project are divided into three parts:

Part A – Quick Test

This test is conducted with an initial concentration of 100 mg NM/L at pH 7. The pH is stabilized with 25 mmol/L MES buffer in 10 mmol/L $NaNO_3$ as a background electrolyte. Three independent batches, each in triplicate, are sampled at the start and after 24 hours. Samples are filtered in 6 mL centrifugal ultrafiltration units within 15 minutes at a minimum of 3000 g, and the target analyte in the filtrate is determined by ICP-MS or ICP-OES. This high initial concentration would permit testing of carbon-based NMs, though this was not addressed in the project. The result after 24 hours provides the operationally defined solubility of the NM at pH 7.

Part B – Extended Test

This test follows the procedure of Part A but uses a lower initial concentration of 10 mg NM/L, includes additional pH values (pH 5 and pH 8), and introduces a temporal component to assess dissolution behavior. Samples are collected at eight time points (0, 0.5, 1, 2, 4, 24, 48, and 96 hours) and analyzed as in Part A. Ideally, the dissolution dynamics should follow first-order reaction kinetics, though, for reasons outlined in the report, this is rarely achieved. The methodology for deriving the dissolution kinetics or rate constant from the results, and the

manner of its presentation, is still under discussion within the relevant OECD committees at the time of this report.

Part C – Flow-Through Test

Established with reference to other studies, primarily within OECD WNT Project 1.05, this test utilizes a PTFE filter holder with 45 mm diameter filter membranes. A 10,000 g/mol MWCO ultrafiltration membrane is loaded with 10 mg of the NM to be tested. The filter holder is flushed for 5 hours with a medium from Part B at a rate of 1 mL/min, and an automatic sample collector collects 5 mL (every 5 minutes) per sampling vial throughout the duration. Every fifth container is used to measure pH, documenting pH stability over the course of the experiment. After approximately two hours, a steady state dissolution should be established. Considering the NM mass dissolved by that point and its surface area (using a sphere model), the dissolution rate can be calculated. An additional variant (Part C2) aggregates the outflow from the filter holder into a single container, allowing determination of the average dissolution rate over the entire 5-hour test period. Here, too, pH is periodically monitored.

A Standard Operating Procedure (SOP) was created for the proposed methods, and an interlaboratory round-robin test involving 17 laboratories was initiated. A one-day training session for interested laboratories was conducted at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), along with two online Q&A sessions via Zoom. Results are partially included in this report and are still being further evaluated at the time of report preparation.

Zusammenfassung

Ziel des Projektes war die Entwicklung einer methodischen Basis zur Erstellung eine OECD Prüfrichtlinie für die Testung von Nanomaterialien (NMs) auf ihre Löslichkeit und Auflösungsrate. Dies umfasste neben dem experimentellen Ansatz auch die relevante Zusammensetzung der Testmedien, in denen die Auflösung von NMs bestimmt werden sollte, der Zeitrahmen, die Beprobungsfrequenz und die Art der Abtrennung von ungelösten NMs von den gelösten Bestandteilen. Die Entwicklung sollte Bezug nehmen auf bestehende und bereits in Entwicklung befindliche Prüfrichtlinien. Ausgangspunkt waren die OECD Richtlinie TG 105 zu *Water Solubility*, sowie das OECD GD 29 zur *Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media.*

Um dieses Ziel zu erreichen, wurden sowohl statische (Batchtest) als auch dynamische (Durchfluss) Verfahren auf ihre Eignung überprüft. Zur Trennung von ungelöstem NM und der gelösten Fraktion wurden im Batchtest Zentrifugal-Ultrafiltrationseinheiten mit 6 mL Probenmenge und 10.000 g/mol (Dalton) MWCO gewählt, sowie im Durchfluss-Test 45 mm Durchmesser Ultrafiltrationsmembranen aus regenerierter Zellulose mit ebenfalls 10.000 g/mol MWCO.

Als wichtigster Parameter, der während des Tests kontrolliert und idealerweise konstant gehalten werden muss, gilt der pH-Wert, da viele Substanzen pH-wertabhänge Löslichkeiten und damit auch Auflösungsraten zeigen. Gleichzeitig beeinflusst die Auflösung von Substanzen in ungepuffertem Medium den pH-Wert, meist durch Verbrauch von Protonen (oder Abgabe von Hydroxydionen). Dies muss entweder aktiv (durch einen Autotitrator) oder passiv (durch Zugabe geeigneter Puffer) verhindert werden. Gleichzeitig sollen Bestandteile des Mediums weder Metallionen komplexieren, noch schwerlösliche Verbindungen mit ihnen eingehen, da sonst das Ergebnis verfälscht würde. Auch die Initiierung oder Katalyse von Redox-Reaktionen sollte ausbleiben. Aus im Bericht dargelegten Gründen wurde auf eine aktive pH-Kontrolle verzichtet. Die zur pH-Stabilisierung eingesetzten Puffer sind ausgewählte, sogenannte *Good's Buffer*, die in der Biochemie entwickelt wurden und eine geringe Metallkomplexierung zeigen. MES wurde für den Bereich von pH 5 und MOPS für den Bereich von pH 7 bis pH 8 ausgewählt. Bei der Auswahl der Medienzusammensetzung wurden keine in natürlichen Wässern vorkommenden Stoffe berücksichtigt, die schwerlösliche Verbindungen eingehen können. Unabhängig von der gewählten Medienzusammensetzung können die entwickelten Tests mit beliebigen anderen Medien verwendet werden, solange sie z.B. mit der Ultrafiltration kompatibel sind und der pH-Wert kontrolliert oder zumindest bestimmt wird.

Die im Projekt entwickelten Verfahren teilen sich in drei Bereiche:

Part A – Schnelltest wird mit einer Startkonzentration von 100 mg NM/L bei pH 7 durchgeführt. Der pH wird stabilisiert durch 25 mmol/L MES Puffer in 10 mmol/L $NaNO_3$ als Hintergrundelektrolyt. Es werden drei unabhängige Batches jeweils in Triplikaten am Anfang und nach 24 Stunden beprobt. Die Proben werden in 6 mL Zentrifugations-Ultrafiltrationseinheiten innerhalb von 15 Minuten bei mindestens 3000 g filtriert und im Filtrat der Zielanalyt mit ICP-MS oder ICP-OES bestimmt. Die hohe Anfangskonzentration würde sogar eine Testung von kohlenstoffbasierten NMs erlauben, dies wurde innerhalb des Projektes aber nicht untersucht. Aus dem Ergebnis nach 24 Stunden ergibt sich die operationell definierte Löslichkeit des NMs nach 24 Stunden bei pH 7.

Part B – erweiterter Test wird durchgeführt wie Part A, jedoch bei geringerer Anfangskonzentration von 10 mg NM/L. Hinzu kommen weitere pH-Werte (pH 5 und pH 8) und eine zeitliche Komponente des Auflösungsverhaltens. Es werden insgesamt an acht Zeitpunkten

(0, 0.5, 1, 2, 4, 24, 48 und 96 Stunden) Proben entnommen und wie in Part A analysiert. Die aus den Ergebnissen ersichtliche Auflösungsdynamik sollte idealerweise einer Reaktionskinetik erster Ordnung folgen, wird dies aus im Bericht dargelegten Gründen aber selten tun. Die Methodik, mit der aus den Ergebnissen die Auflösungskinetik oder die Ratenkonstante abgeleitet wird und in welcher Weise sie dargestellt wird, ist zum Zeitpunkt der Erstellung dieses Berichts noch Teil einer Diskussion innerhalb der zuständigen Gremien der OECD.

Part C – Durchflusstest wird in Anlehnung an andere Arbeiten, hauptsächlich innerhalb des WNT Projekts 1.05 der OECD, etabliert. In einem PTFE-Filterhalter für Filtermembranen mit 45 mm Durchmesser wird eine 10.000 g/mol MWCO Ultrafiltrationsmembran mit 10 mg des zu testenden NMs beaufschlagt. Der Filterhalter wird für 5 Stunden mit einem Medium aus Part B mit 1 ml/min durchströmt und ein automatischer Probensammler sammelt jeweils 5 mL (alle 5 Minuten) pro Probenahmegefäß über den gesamten Zeitraum. In jedem fünften Gefäß wird der pH-Wert ermittelt, um die Konstanz über den Versuchsverlauf zu dokumentieren. Nach etwa zwei Stunden sollte sich ein Fließgleichgewicht eingestellt haben. Unter Berücksichtigung der bis dahin durch Auflösung verlorenen NMs und ihrer Oberfläche (Kugelmodell) kann die Auflösungsrate ermittelt werden. Eine weitere Variante (Part C2) aggregiert den Auslauf des Filterhalters in einer einzelnen Flasche und bestimmt nach fünf Stunden die mittlere Auflösungsrate über den gesamten Testzeitraum. Auch hier wird periodisch der pH-Wert kontrolliert.

Für die vorgeschlagenen Verfahren wurde eine Standard Operating Procedure (SOP) entworfen und ein Ringtest mit 17 Laboren initiiert. Es wurde ein eintägiges Training der daran interessierten Labore in den Räumlichkeiten des Fraunhofer Instituts für Molekularbiologie und Angewandte Ökologie (IME) durchgeführt und zwei Fragestunden online über Zoom abgehalten. Die Ergebnisse sind im Bericht auszugsweise dargestellt und werden zum Zeitpunkt dieser Berichtserstellung noch weiter ausgewertet.

1 Introduction

The environmental risk assessment of nanomaterials (NMs) requires detailed and quantitative information about the behaviour and fate of NMs in the environment. Environmental behaviour and fate processes which NMs undergo influence both, the exposure and hazard.

Four major transformation processes have been identified which predominantly control NMs fate:

1. Dissolution
2. Chemical transformation
3. Biological transformation
4. Homo- and hetero-agglomeration

NMs may undergo one to all four processes simultaneously or sequentially. From the perspective of a single nanoparticle (NP):

Dissolution leads to the release of ions or molecules and the NP loses mass and eventually vanishes. Dissolution of a NP is accompanied by shrinking and therefore loss of surface area. The ratio of surface area to mass loss is depending on the shape of the particle. Partial dissolution might be observed when chemical transformation leads to e.g. a protective coating or the NP consists of different materials, like e.g. mixed oxides with different solubilities of the different compounds making up the particle.

Chemical/biological transformation may induce a change in composition, size, shape, mineralogy and with this a change in behavior and toxicity, but the nanoparticulate character is preserved. Transformation can take place on/in the particle itself or by dissolution and re-precipitation. Dissolution is an important initial step in many transformation reactions, however it does not lead to an ion release. Overcoating of a NP by (macro-)molecules stemming from the medium in which the NP is dispersed, is also a transformation reaction. This is often termed (bio-)corona formation and involves humic substances in natural waters but also proteins, polysaccharides and others with affinity to surfaces.

Agglomeration processes lead to a loss of the original nanoparticulate character by formation of particle associations. The resulting particle agglomerates may remain in the nanoparticle size range or grow to clusters >> 100 nm. While the characteristic of the original NM in terms of particle size is lost, the agglomerates still contain the original pristine or chemically/biologically transformed NM with the potential to be released at a later stage. It has become common practice to distinguish agglomerates and aggregates, where agglomerates are particle associations where the particles are held together by weak forces like electrostatic or Van der Waals forces, while aggregates are held together by strong forces like chemical bonds. While it can be debated how these two states can be distinguished, the formation of an agglomerate from single particles firstly leads to associations held together by weak forces and therefore the term “agglomeration” appears the correct one for this process.

Complete dissolution is the pathway that leads to the loss of the NP and the release of (potentially hazardous) ions or molecules and can be seen as a NP (and eventually NM) degradation process with the evolution of metabolites. This process is relevant for NMs behavior and lifetime in the environment and for testing the NMs behavior and toxicity in regulatory tests.

During the OECD expert meeting in Berlin in January 2013 (OECD 2014) experts agreed on the necessity to develop two new OECD Test Guidelines (TG) and a corresponding guidance document (GD) enabling the testing on dissolution behavior and the agglomeration/dispersion

stability of NMs in relevant environmental aquatic media. The developed TGs shall provide the quantitative and validated experimental methodology to determine the NM's solubility and agglomeration/dispersion stability in environmental aquatic media.

In 2017 the TG 318 *Dispersion stability of nanomaterials in simulated environmental media* was accepted and published by OECD (OECD 2017) with an accompanying GD *for the testing of dissolution and dispersion stability of nanomaterials and the use of the data for further environmental testing and assessment strategies* published in 2020 (OECD 2020) which inter alia already includes some initial guidance on the endpoint dissolution (rate). However, the parallel development in OECD WNT Project 3.10 *Determination of the dissolution of metal nanomaterials in aquatic media* had not come to a conclusive TG development in 2019.

In 2019, funded by and on the behalf of the German Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection, this project was launched at the Department of Environmental Geosciences of the University of Vienna and in collaboration with the Fraunhofer Institute for Molecular Biology and Applied Ecology IME, supervised by the German Environmental Protection Agency aiming to support finalization of OECD WNT project 3.10. As part of this process WNT project 3.10 was renamed *Test guideline on dissolution rate of nanomaterials in aquatic environmental media* (OECD 2024).

The objectives were:

a) A set of universal, cost-effective and reproducible methods to determine NM solubility and dissolution rate

b) Simplicity, with the aim to be performed in regular commercial laboratories

c) Applicability of the methods to MN dispersions and powders

d) Identification of the different sources of error in the procedures and their impact on the methods accuracy

e) Definition of the method parameters: NM concentration or amount, ultrafilter cut-off, media composition and test duration.

As a starting point the method development utilized the existing documents *TG 105 Water Solubility* (OECD 1), *GD 29 Guidance Document on Transformation & Dissolution of Metals and Metal Compounds in Aqueous Media* (OECD 2), *GD 318 for the Testing of Dissolution and Dispersion Stability of Nanomaterials, and the Use of the Data for Further Environmental Testing and Assessmen*t (OECD 3) and the draft document (unpublished) developed earlier in WNT project 3.10 *Guideline for Determining the Dissolution of Metal Nanomaterials in Aquatic Media*. Developments in the OECD WNT project 1.05 *Guidance Document on Determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biological media* were constantly considered as soon as updates on the developments became available.

2 Background

The determination of dissolution, solubility and dissolution rate of solids in liquids is a core competence of physical chemistry, mineralogy, geochemistry and soil and pharmaceutical science and a wealth of literature is available. Solubility products of hundreds of compounds are available, while a comprehensive "complete" list is still lacking. Geochemical computer models as PhreeqC or Visual Minteq (to name only two) allow to calculate dissolved concentrations and the aqueous speciation of a range of solids and minerals in a wide variety of water chemistries. However, parameters like solubility or dissolution rate are often determined in specialized experiments, adapted to the solid material in question, using analytical techniques, which are not commonly available in standard laboratory settings (e.g. voltammetry).

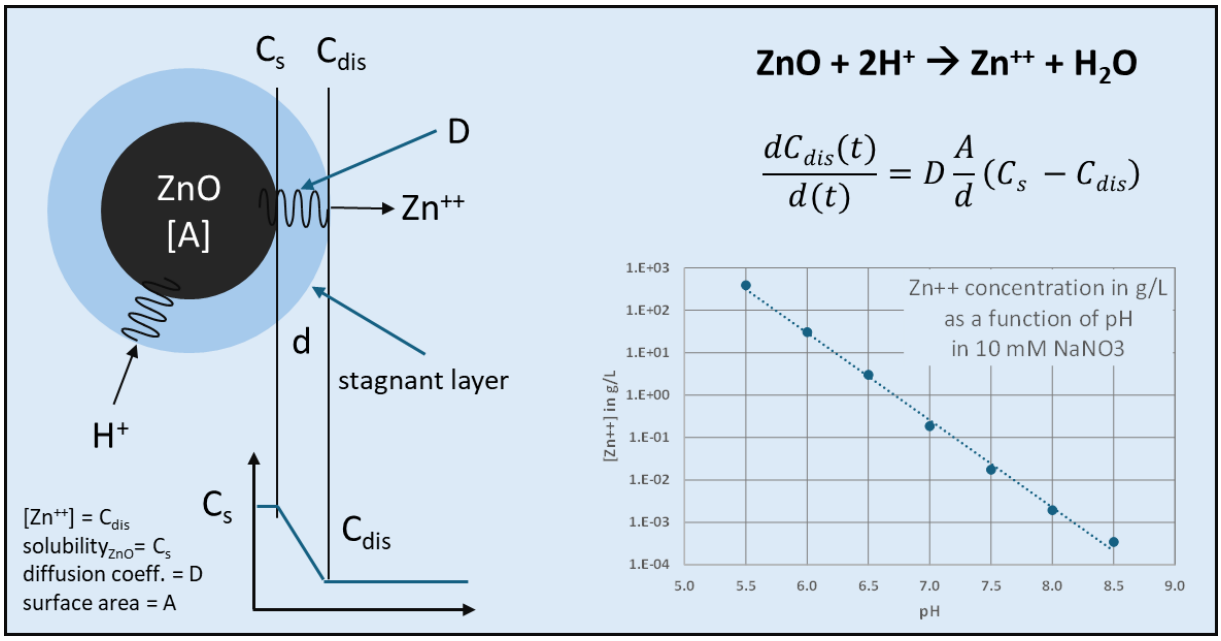
Critical in the determination of solubility and dissolution rate of NMs is the required separation of the solute (dissolved compounds, ions) in the medium and the solid NM. This can be achieved instrumentally, by using ion-selective electrodes or variants of electrochemical techniques (polarographic analysis) or by physical separation (filtration, centrifugation, dialysis, ion exchange). The electrochemical methods have the advantage that they require only short times for the measurements and can be conducted directly in the media, with no additional handling like filtration required, what prevents the risk of losses or contamination, especially at low concentration of the solutes. However, they can only be applied to a limited number of elements and, in the case of ion selective electrodes, might lack the required sensitivity and/or precision. In the case of NMs, filtration and centrifugation must address the presence of ultra-small particles and ultra-filtration, or ultra-centrifugation are required, adding costs and time (ultrafiltration) or equipment costs (ultracentrifugation).

Determination of solubility requires the achievement of equilibrium between the solid NM and the solute in a liquid medium. This is relatively easy to obtain in stirred or shaken batch experiments. It will, however, require extended periods of time for low soluble materials. Schmidt and Vogelsberger (2009) investigated in their excellent study the dissolution of various TiO_2 (nano)materials. Depending on the pH, the equilibrium was reached after ~ 400 - 600 h with clear signs of oversaturation (concentrations of the solute above the equilibrium) in the first ~ 20 - 200 h. The authors attributed this to a kinetic size effect, inherent with NM, and concluded that < 70 h a too high solubility could be reported, while after 500 h a correct value within the accuracy of the method, can be obtained.

In general, dissolution occurs as a two-step process: the first step is the reaction at the surface of the particle, which releases the compound (ion, molecule) into the medium and the second step is the diffusion of the compound through the solid-liquid interface into the medium. This interface is typically a layer of stagnant water. This process is schematically shown in figure 1 on the example of a zinc oxide particle. The concentration of the dissolved zinc ions at the surface of the particle equals the solubility of zinc oxide under the conditions of the medium composition and temperature at the surface (C_s). On the other hand, the concentration at the transition from the stagnant layer to the bulk medium equals the actual concentration at the specific point in time (C_{dis}). As long as $C_{dis} < C_s$ the concentration gradient forces zinc ions to diffuse through the stagnant layer into the bulk medium. With increasing C_{dis} and decreasing concentration gradient the process slows down. When the bulk medium concentration equals the solubility of the zinc oxide an equilibrium is reached, the concentration in the bulk now equals the solubility of zinc oxide under the given conditions (medium composition, temperature). Zinc oxide is a special but often encountered case here, since the hydrolysis at the surface of the particle produces hydroxyl ions. This affects not only the pH at the surface of the particle, because the produced hydroxyl ions increase the pH locally, but also requires protons if the pH in the bulk solution

should be held constant. The production of hydroxyl ions also makes the dissolution and with it the solubility of zinc oxide strongly pH-dependent.

Figure 1: Principle of the dissolution process with zinc oxide as an example and pH-dependence of the ZnO solubility



Source: own graph, calculations done with Visual Minteq

From figure 1 and the shown Noyes-Whitney equation the main controlling parameters, which influence the solubility and the dissolution rate become apparent. The solubility is determined by the NM itself, its composition, the composition of the surrounding medium, and especially, for those dissolution reactions which involve protons/hydroxyl ions, by the pH. Temperature also plays a role, usually increasing the solubility with temperature. Another factor, not depicted in figure 1, is the free ion activity of the solute. With complexing agents in the media (e.g. natural organic matter or EDTA), the free ion activity of the solute (here Zn^{++}) may be decreased by complexation, leading to a higher total concentration ($C_{tot} = C_{dis} + C_{complexed}$). Also, when ions of a different kind (in our case non-Zn ions) are present, the free ion activity of Zn^{++} would be decreased, leading to a higher C_{dis} concentration. This underpins the necessity of a defined and constant pH and a defined concentration of a background electrolyte.

For the dissolution rate $dC_{dis}(t)/d(t)$ the situation is slightly more complex. The solubility C_s of the NM in the respective medium is still the major parameter. Additionally, the available surface area of the NM (A), the concentration gradient between the NM surface and the bulk medium (C_s, C_{dis}), the thickness of the stationary layer (d) and the diffusion coefficient of the dissolved compound in the medium (D) play a role. The fact, that the dissolution rate decreases with the increase of the concentration in the bulk medium (C_{dis}) means, that we must distinguish between methods, in which this concentration is allowed to build-up (e.g. batch experiments) what will gradually slow down the dissolution rate and infinite sink approaches where C_{dis} is kept at a minimum or ideally at zero (e.g. continuous flow, ion exchange). Considering environmental situations, NMs will reach only low concentrations in e.g. surface waters (rather ppb than ppm). In most situations NMs will dissolve into an almost infinite volume of water with only traces of their components already present as a solute (exceptions might exist). In a batch experiment, only the earliest stage of dissolution will represent the dissolution rate the NM will show in the real world.

With these influencing factors, the dissolution rate becomes dependent on the experimental design. The thickness of the stationary layer is depending on the shear forces around the particle. Shaking or stirring and the speed of the respective agitation method will influence *d* and the rate of dissolution. Also, the surface area *A* of NMs in the experiment and with this the amount of NM, or better, the solid to liquid ratio, influences the dissolution rate.

It therefore becomes clear that dissolution rates given as a concentration increase of the solute over time (e.g. $mg*L^{-1}*s^{-1}$) only describe the dissolution behavior of the specific material for a specific experimental condition and a normalization to the NMs surface area, as employed in the experiment ($mg*L^{-1}*s^{-1}*m^{-2}$ or in terms of mass released $mg*s^{-1}*m^{-2}$), is more universal. In the case the surface area of the NM is unknown, the mass of the NM used in the experiment could be used ($mg*L^{-1}*s^{-1}*g^{-1}$ or in terms of mass released $mg*s^{-1}*g^{-1}s$) the latter being a mass fraction of NM dissolved over time what could also be expressed as percentage of NM dissolved over time.

The dependency from the surface area *A* leads to the situation, that NMs with a (broad) size distribution show different dissolution rates over the course of dissolution. During the initial period smaller particles with higher specific surface area dissolve faster than the larger ones. With proceeding dissolution, the smaller particles shrink or vanish, what will slow down the dissolution process. A clear first order reaction kinetic will therefore only be observed with monodisperse NMs. A solution to this problem is to take the reduction of particles size and the related loss of surface area into account. Examples for this can be found in Cardoso et al. (2022) for the dissolution of ZnO and Song et al. (2023) for various NMs. Both approaches assume a spherical particle of a defined size and neglect effects from NM agglomeration or NM aggregates. Since most NMs will show a size distribution and non-spherical shape in various states of agglomeration/aggregation these approaches are difficult to introduce into a general model.

If very small particles (~ < 20 nm) are present, those might exhibit a higher solubility than the rest of the sample in the experiment, (kinetic size effect: Schmidt and Vogelsberger (2009)), further complicating the interpretation of the results.

When normalized to surface area or mass, the resulting dissolution rate should be independent of the initial amount of sample. However, in a batch experiment the solid to liquid ratio will influence the build-up of C_{dis} and with lower ratios also higher dissolution rates are expected. This was demonstrated in continuous-flow experiments by Keller et al. (2020) for a broad set of NMs. For the determination of dissolution rates the choice of a rather small amount of sample, keeping the concentration C_{dis} far below the solubility of the NM, seems advisable for a continuous-flow and a batch test, simulating realistic environmental conditions. For well-soluble NMs this bears the risk of material depletion within the time span of the experiment. On the other hand, for the determination of solubility, it is required to have solid material left over after the test duration to obtain a correct value in (ideally) equilibrium.

Considering these boundary conditions for the aim of creating a universal, simple and cost-effective methodology to test NMs solubility and dissolution rate four different experimental approaches have been developed:

1. Part A: a quick batch test that delivers the apparent solubility of a NM after 24h at pH7 when the solubility of the NM is below 100 mg/L
2. Part B: an extended batch test that delivers the apparent solubility for a NM after 96h at pH 5, 7 and 8 when the solubility of the NM is below 10 mg/L and the dissolution rate for slow to medium-fast dissolving NMs as long as NM depletion or saturation is not reached before 4h.

3. Part C1: a continuous-flow or flow-through test as an infinite sink approach. Regular sub-sampling in defined time intervals. Simulating natural conditions with very low solid to liquid ratios.
4. Part C2: a continuous-flow or flow-through test as an infinite sink approach. Integrating sampling. Outflow is collected and one sample is taken from the collection bottle after a defined time. Simulating natural conditions with very low solid to liquid ratios.

3 Development of methods for regulatory testing of nanomaterials on their solubility and dissolution rate

When the project started, the initial approach was to use smallest initial NM concentration in the experiments, typically 1 mg/L or less. This was decided with the objective to provide most realistic conditions with low solid to liquid ratios. During the project, several drawbacks of this approach became apparent:

1. When working with NM powders, the weighing of the small NM amounts (0.01 to 0.1 mg) is difficult and requires measures against electrostatic effects as well as either a balance with µg-resolution or large volumes of media. While this is no problem in a research laboratory, it could prevent the implementation of the method in a commercial laboratory.
2. Producing stable dispersions from NM powders to overcome the weighing problems is cumbersome and leads to a premature dissolution of NMs.
3. Precise and reproducible dosing such small amounts of NM is difficult when the NM is agglomerated.
4. Losses of dissolved ions to vessel walls and filtration membranes require precautionary measures. Tests showed about 50 % metal ion loss from a 200 µg/L zinc and copper nitrate solution to (not pre-treated) ultrafiltration membranes.

Over the course of the project, the use of a 100 mg/L initial concentration was brought up from several sides and especially with regard to Part A (solubility after 24h) the higher starting concentration made sense to obtain equilibrium conditions also for better soluble materials. Still, the experiments with lower concentrations were useful in localizing the sources of error and defining the media composition.

3.1 Media composition and experimental framework

3.1.1 Media composition

As in the development of OECD TG 318 *Dispersion Stability of Nanomaterials in Simulated Environmental Media* it was the objective to employ environmentally relevant media. In contrast to realistic, relevant means to employ only those components in the medium, which have an influence on the reaction of interest and are widespread present in natural waters in concentrations which can influence the reaction.

Controlling parameters and components are temperature, pH, presence of complexing agents, overall electrolyte concentration (ionic strength) and components which might lead to transformation into less soluble forms of the NM. The latter most likely are ortho-phosphate, sulfate, carbonate, chloride and sulfide which all can form salts of low solubility.

Ionic background. Since the test on NM transformation was not in the scope of this project and the environmental concentrations are typically low compared to the finally adopted initial concentrations of NM, ortho-phosphate, sulfate, chloride and sulfide were omitted. Their effect would have been marginal. This, of course, does not prevent the user of the developed methods to employ a more complex medium for the testing and deviate from the SOP, if it is compatible with the test design. Bicarbonate was initially kept as a natural pH-buffering agent at concentrations between 10 and 25 mmol/L, but later reduced to 5 mmol/L because it showed negative effect in combination with the chosen biochemical pH-buffering systems at pH <7 and with the higher initial NM concentrations of 100 or 10 mg/L. With bicarbonate the proton-consuming dissolution reactions of e.g. ZnO raised the pH more than without bicarbonate. As a background electrolyte $NaNO_3$ at 10 mmol/L (850 mg/L) was selected. Although NaCl would

have more environmental relevance, because of the higher natural abundance of Cl^-, but chloride ions could interact with NMs and the dissolved ions. The chosen ionic strength is in the upper range of what is found in most natural waters but still 1/10 of what is typically used as background electrolyte in dissolution experiments (Schmidt and Vogelsberger (2009), Jiang et al (2015)).

Complexation. Regarding complexing agents, natural organic matter (NOM) (dissolved organic carbon, DOC) is the most important component in natural waters. Complexation of metal ions will reduce the free ion activity of the metal and therefore allow a faster dissolution and a higher solubility (with respect to total metal concentration). The effect of NOM is however not unilateral. With realistic concentrations of NOM (~ 10 mg/L = 5 mg/L DOC) and initial concentrations of the NM at 100 mg/L also here effects are expected to be marginal. Also, if NOM would be employed in the tests, a standard, purified NOM would be required for comparability. This NOM would be required in substantial amounts but the availability of standard, purified NOM from e.g. the International Humic Substance Society (IHHS) is limited. Clean-up procedures for commercially available, mostly lignin-derived NOMs are cumbersome and even the purified NOM will differ in quality and composition from those found in natural waters. Table 1 shows the cation concentrations found in Suwannee River Natural Organic Matter standard (SRNOM) and a non-purified commercial NOM (Sigma Aldrich Humic Acid).

Table 1: Elemental analysis of Suwannee River NOM and Sigma-Aldrich Humic Acid (HA) (mg of ions per 1g of DOC)

	Ca	Mg	Na	K	Fe	Ni	Cu	Mn	Co
SRNOM	1.4 +/-0.6	0.17 +/-0.01	316 +/-3	5.9 +/-0.6	8.8 +/-1.3	<0,01	0.05 +/-0.04	<0,01	<0,01
Sigma-HA	92.2 +/-10	6.1 +/-0.1	698 +/-4	55.3 +/-0.3	40.8 +/-2	0.33 +/-0.03	0.3 +/-0.05	0.03 +/-0.01	0.07 +/-0.01

SRNOM: Suwannee River Natural Organic Matter, HA: humic acid. (Concentrations of ions per 1g of DOC). Source: UBA Texte 108/2017: Clarification of methodical questions regarding the investigation of nanomaterials in the environment.

NOM is also a natural pH buffer and could assist in the stabilization of the pH. At the higher initial NM concentrations, the effect would however again be marginal.

It has therefore been decided to omit NOM from the final media composition.

Control of pH. Several factors influence the pH in the media during NM dissolution. Some NMs, especially metal oxides like ZnO, consume protons during the dissolution process. And at pH > 5 the uptake of atmospheric CO_2 will increase the pH. Since solubility is often pH-dependent (see figure 1 for ZnO with differences of two orders of magnitude per one pH unit) the pH must be precisely controlled during the experiment.

The two major ways to stabilize the pH are an active control with pH measurements and automatic acid and base titrations in a stirred batch reactor and the use of chemical buffers. The autotitration with NaOH and HNO_3 has the advantage of adding only Na^+ or NO_3^- as electrolytes to the test medium, which are already present from the background electrolyte. Also, the pH control is precise to about 0.1 pH unit. Narrowing the pH range to less than +/- 0.1 pH unit can lead to back-and-forth titrations where the two channels of the titrator correct each other reciprocative. In any case, the volume of the medium is not kept constant. The method is relatively cost-intensive because of the equipment costs, especially when multiple systems are required to enable parallel replicate analyses. In OECD WNT project 1.05 *Guidance Document on*

Determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biological media an SOP for testing NM solubility and dissolution rate in a pH-controlled, stirred batch reactor is developed. Therefore, the work in this project was focused on a different set-up.

pH-Buffers are a simple and cost-effective way to keep pH-values in a certain band. To be applicable for the purposes of the tests developed in this project, most of the traditional buffer systems (phosphate, acetate, citrate, borate) cannot be applied due to possible influence on the dissolution reaction. The buffer should not complex metal cations, not be redox active and not lead to precipitation of low-soluble metal compounds. Biochemical buffers (so called Good's buffers) have been developed since the 1960s and many of them fulfill these criteria (Ferreira et a. 2015). Buffering capacity is highest around the pKa-value of the buffer and effective at pKa +/- 1 pH-unit. Among the existing Good's buffers the MES buffer has the lowest pKa value of 6.16 (20°C) and CABS 10.70 (20°C) the highest, what limits the pH region which could be covered to values between ~ pH 5 and ~ pH 11. When working at low initial NM concentrations of 1 mg/L or less (representing µmolar concentrations of the NM), 10 mmol/L of the biochemical buffers should be able to keep the pH constant even with dissolution reactions which consume protons and small drifts should be reproducible in-between experiments of the same NM if NM and buffer concentrations are reproducible.

The later adopted higher initial NM concentrations of 100 and 10 mg/L do exceed the buffer capacity if protons are consumed and the NM is well soluble (e.g. with metal oxide dissolution at lower pH). An increase of the buffer concentration by a factor of 10 or 100 is not advisable because of possible effects on the dissolution reaction or increased tendency for the NM to agglomerate (total background concentration rises to > 0.1 mol/L). As a compromise a concentration of 25 mmol/L of the buffer has been chosen and a precise monitoring of the pH during the test is necessary.

In European surface waters 75% of the pH-values lie between pH 5 and pH 8, with another 1% being below pH 5 and 22% being between pH 8 and 8.5 (Salminen 2006). With many compounds showing a higher solubility at lower pH and the MES buffer reaching down to maximum pH 5, pH 5 has been chosen as lower limit of the test media. The upper limit has been set to pH 8 where the MOPS buffer still offers sufficient capacity with a pKa of 7.2. Both buffers (MES and MOPS) have been documented to not complex metal cations and not produce free radicals (Ferreira et al. 2015 and literature cited therein), hence should not be redox active. With this they are unlikely to influence the dissolution process. If the upper limit should be increased to pH 8.5 to harmonize with other guidelines, HEPBS could be employed, this buffer was found to be equally inactive as MES or MOPS. However, while MES and MOPS overlap in their buffering ranges, this is not the case for MES and HEPBS and for tests between pH 5 and 8.5 MOPS would be required and this would then introduce three different buffering systems across the testing.

The use of $NaHCO_3$ has been discussed controversially. It is present in almost all natural waters with a median concentration of ~ 2 mmol/L in European freshwaters (Salminen 2006). In dissolution experiments with low initial NM concentrations and 10 mmol/L of biochemical buffers MES and MOPS it served as an natural background electrolyte and additional pH-buffer, but at higher initial NM concentrations it became apparent that it reduces the buffering properties of MES at pH 5. It has been first reduced to 5 mmol/l in pH 5 tests but later been omitted in all tests. This makes the preparation of the media easier and improves pH stability at pH 5.

The finally adopted media compositions are given in table 2.

Table 2: Finally adopted media compositions for batch and continuous-flow experiments (in mmol/L) indicating changes made from experiments with low initial NM concentration (≤ 1 mg/L) to high initial concentration (10 or 100 mg/L)

	MES buffer[a)]	MOPS buffer[b)]	$NaNO_3$	$NaHCO_3$
pH 5	10 → 25	0.00	10	5 → 0
pH 7	0.00	10 → 25	10	10 → 0
pH 8	0.00	10 → 25	10	25 → 0

a) Commercial ready-made buffer solutions adjusted to pH 5

b) Commercial ready-made buffer solutions adjusted to pH 7 and pH 8

3.1.2 Nanomaterials used for testing

The selection of NMs for the test development was based on literature availability, solubility products, availability in geochemical speciation models and discussions during the meetings with the Joint Expert Group (JEG) on environmental fate and ecotoxicity testing of the OECD WNT and WPMN. The mixed Samarium Strontium Cobalt Oxide was introduced because of a request for a more heterogeneous NM. For this material no literature data exist. Table 3 lists the selected materials and their properties.

Table 3: Selected nanomaterials for test development and validation

Type	Material	Dissolution behavior	Specific properties
ZnO[a)]	NM110[b)]; Sigma < 100 nm dispersion	Readily soluble < pH 7.5	Highly dependent on pH, consumes protons in dissolution, broadly covered in literature, solubility of the bulk material available
Ag	NanoComposix NanoXact 80 nm dispersion; & Sigma < 150 nm powder	Low to moderate soluble under neutral pH due to formation of protective layer, transformation product Ag_2O readily soluble	Requires oxidation, may be partially soluble in certain cases (formation of protective coating), broadly covered in literature
CuO	PlasmaChem Berlin 40 nm powder	Moderate soluble	Highly dependent on pH, consumes protons in dissolution, well covered in literature, solubility of the bulk material available
$BaSO_4$[a)]	NM 220[b)]	Moderate soluble	Solubility largely pH independent, does not affect the buffer system, well studied, solubility of the bulk material available
$Sm_{0.5}Sr_{0.5}Co_2O_x$[a)]	Sigma Aldrich < 50 nm	Moderate soluble	Mixed oxide, shows partial dissolution, shows heterogeneous dissolution, no literature available

a) Selected for round robin, b) JRC Nanomaterials Repository (https://joint-research-centre.ec.europa.eu/scientific-tools-and-databases/jrc-nanomaterials-repository_en)

Some of the materials are available as stable dispersions, which made experiments with low initial concentrations relatively easy to conduct. Stabilizing agents do not interfere, because the apparent solubility or/and dissolution rate was determined, and the focus was more on the effect of sample handling, pH and the type of buffers used on the outcome of the experiments.

When working with powders (ZnO & CuO) it became apparent, that for experiments at low initial concentrations a stable dispersion with known NM concentration must be prepared first. Weighing such small amounts (≤ 1 mg) directly into the batches poses some difficulties which can diminish the reproducibility. Also, in the former work on OECD WNT Project 3.10 (i.e. before the start of this research project), the NMs were required to be in a stable dispersion before starting the dissolution testing. The preparation of stable dispersions from the ZnO and CuO powders was tedious, with pre-dissolution of the NM and removal of larger particles or agglomerates/aggregates. The dispersion did not reflect the NM powder composition in the end. It is also likely that several powder NMs will not form a stable dispersion. It must be considered that the agglomeration/aggregation state of the tested NM will have an influence on the dissolution rate. Exchange with the water trapped in-between NPs of an agglomerate will be slower, leading to elevated solute concentrations in the pores and slower exchange with the bulk medium. With not fully de-agglomerated NMs the apparent dissolution rate will be decreased and the time until a dissolution equilibrium is reached (solubility limit) will be increased (Cardoso et al. 2022).

3.1.3 Initial concentrations or loadings

Batch tests part A and B. As already discussed in the former sections, the use of small initial NM concentrations (≤ 1 mg) was abandoned during the development of the test. Although having advantages due to the low solid/liquid ratio, easier to maintain pH, and extensively used in experiments with Ag-NP dispersions, it appeared impractical as soon as NM powders are tested.

A compromise between easy handling and sufficient NM amount for solubility testing on the one hand and lower solid/liquid ratios for dissolution rate testing on the other was established in the discussions with the OECD JEG. 100 mg/L was set for the quick test on solubility after 24 h (Part A) and 10 mg/L for the extended test (Part B) delivering the solubility after 96 h and the dissolution rate for not too fast dissolving NM.

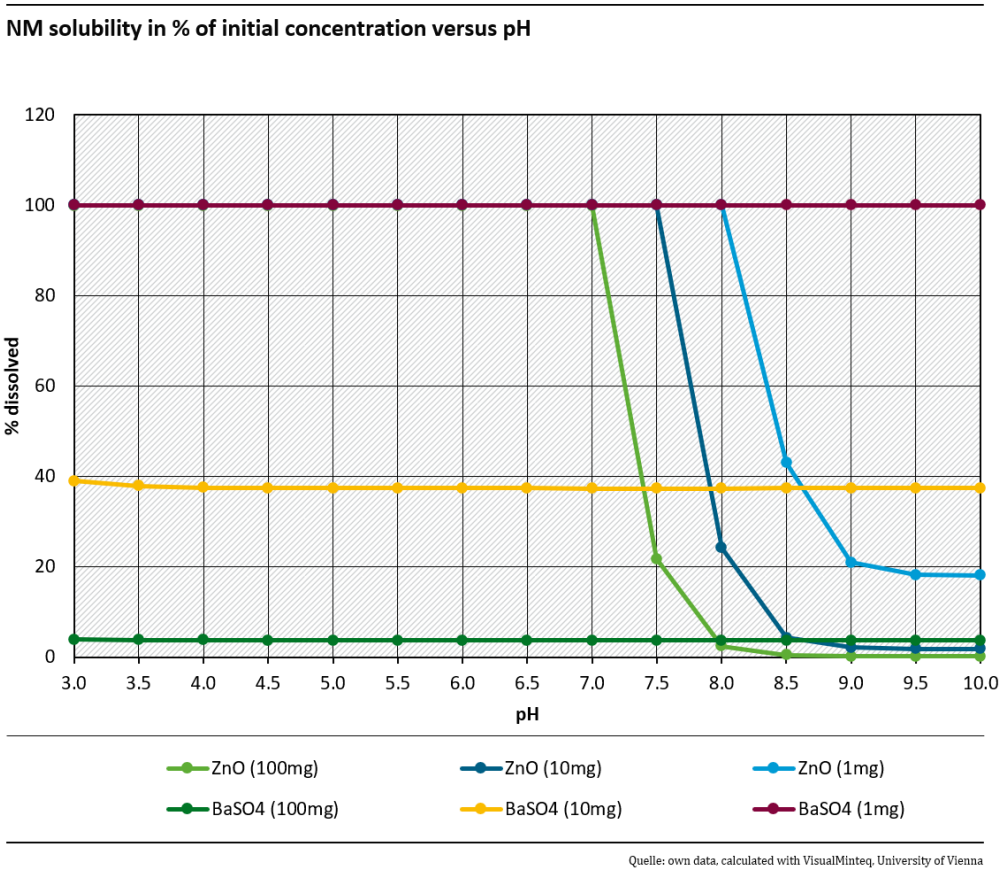
These initial concentrations e.g. 100 mg/L translate into 1.23 mmol/L for Zn^{2+} from ZnO and 2.46 mmol/L OH^- being produced ($ZnO + H_2O \rightarrow Zn^{2+} + 2\ OH^-$) at full dissolution, contrary to µmolar values at 1 mg/L. This requires higher concentrations of the biochemical buffers and the selection of buffers is restricted to their optimal pH range. In principal, this would also mean that the lower pH testing limit would be 5.5 with the MES buffer and small pH increases have been observed with MES, pH 5 and fully dissolving 100 mg/L ZnO. This limit has still to be discussed.

The requirement for the analytical techniques is also reduced at the higher initial NM concentrations. It was agreed that the limit of quantification should be 0.1 % of the max. concentration. This translates to 0.1 and 0.01 mg/L and is for many metals and some non-metals achievable by ICP-OES. With some special precautions even total organic carbon (TOC) analyses might become possible, opening the test for carbon-based materials.

Figure 2 shows the percentage values of introduced NM being dissolved at a certain pH on the examples of ZnO and $BaSO_4$, calculated with the geochemical speciation program Visual Minteq. The calculated solubility for ZnO and $BaSO_4$ at pH 7 and 10 mmol/L $NaNO_3$ is 232 mg/L and 3.73

mg/L respectively. For ZnO, which is readily soluble below pH 7.5, the introduced material will be completely dissolved at all initial concentrations. This of course prevents a correct solubility limit determination, since the whole solid ZnO is dissolved. Another point to stress is, that depending on the initial NM concentration employed, the "percentage NM dissolved" number will vary above pH ~7. A hypothetical threshold of >40% of NM dissolved will be reached with ZnO at pH < 8.6 (1 mg/L), pH < 7.9 (10 mg/L) and pH < 7.35 for 100 mg/L initial NM concentration. For $BaSO_4$, which dissolves widely independent of pH only the 1 mg/L test will exceed the threshold value.

Figure 2: Solubility as percentage of NM dissolved in dependency from the initial concentration in mg/L and pH. Examples are ZnO (zincite) and $BaSO_4$ (baryte) as pH-dependent and non-pH-dependent materials respectively

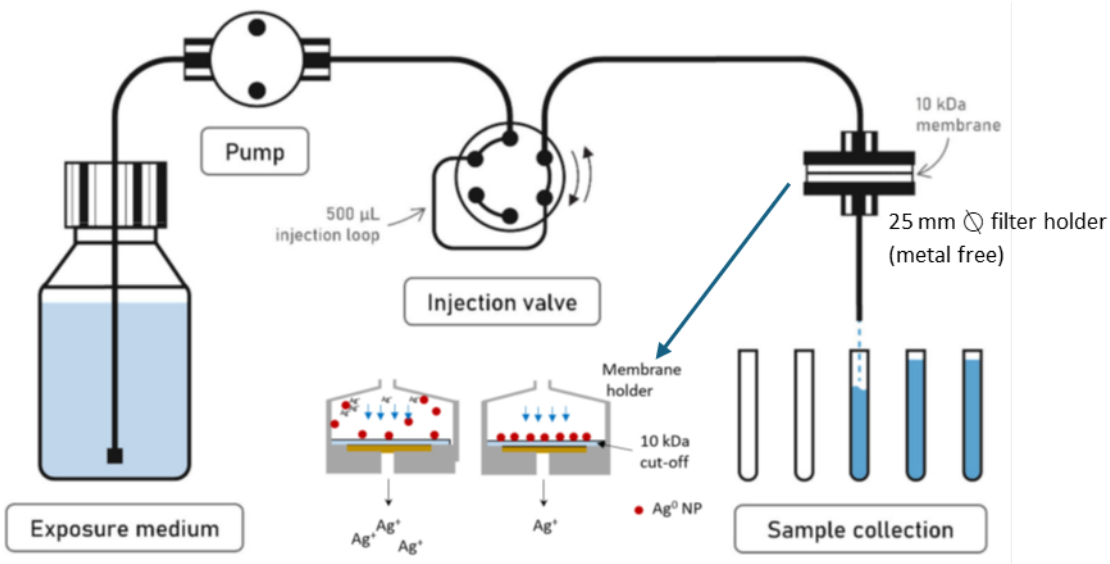


This renders a reporting parameter "percent NM dissolved" in static batch tests useless, as long as other parameters of the experiment are not considered. Simply the change of the initial amount or concentration can change this value (Plakhova et al. 2016).

Continuous-flow Test Part C. A continuous-flow test had been adapted from ISO 19057:2017 and applied by Koltermann-Juelly et al. (2018) to determine the dissolution rates of a wide range of NMs under conditions prevalent in human lungs using a phagolysosomal simulant as medium. The ultrafiltration membrane holder had a diameter of 47 mm and a membrane with an MWCO of 5 kDa, the initial amount of NM was 1 mg and the flow rates in the lower ml/h range. With such low flowrates, the concentrations of solutes in the flow cell reached 18 mg/L for ZnO, what is unrealistic for environmental situations.

To become more realistic for environmental scenarios (low solid/liquid ratios) a further adapted system was developed (Figure 3, Stetten et al. 2022).

Figure 3: Schematic of the setup used for the continuous flow dissolution experiments for stable dispersions and low solid/liquid ratios



Source: Stetten et al. 2022, open access graphic (modified), University of Vienna

A metal-free filter holder of 25 mm diameter containing a 10 kDa MWCO membrane was continuously fed with media from an HPLC pump. An injection valve with sample loop allowed the injection of 500 µL to 2 mL samples (dispersions) into the flow. The NPs would then be transported into the filter holder and deposited on the membrane. With this a uniform distribution of NPs as a monolayer on the membrane was achieved. The loadings of NPs on the membrane were between 1 and 10 µg total and with a flowrate of 60 ml/h the contact time of medium and particles lay in the milli-second range leading to outflow concentrations (with Nanocomposix NanoXact 80 nm Ag-NPs) below 5 µg/L (Stetten et al. 2022) . While these conditions almost represent an infinite sink condition and are closer to the situation NMs will experience in environmental settings, also critical problems were encountered:

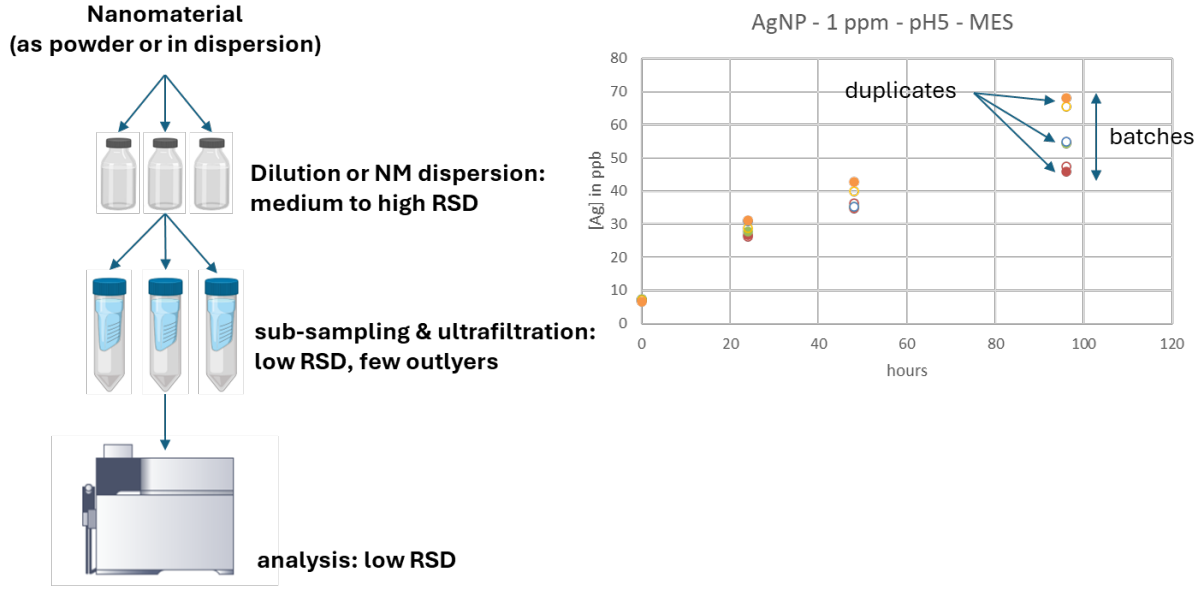
1. With more soluble NMs than the Ag-NPs used in the proof of principle study, the small amounts of NMs deposited on the membrane will be quickly exhausted and fully dissolved.
2. It was observed that it took up to 5h to reach a steady-state dissolution condition. The reason for this could not be identified, but it is likely, that particles remained floating free in the dead-volume of the filter holder and did not deposit on the membrane.
3. As with the batch experiments, the use of powder NMs in this setup would require the preparation of a stable NM dispersion.

Because of these limitations and to align the experimental setup with the developments in OECD WNT project 1.05, it was decided to use larger filter holders (47 mm), where the NMs could be injected as a dispersion or placed as a powder on the membrane. Since this setup works with much higher flow rates than described in Koltermann-Juelly et al. (2018) it was decided to set the NMs loading (powder) to 10 mg.

3.1.4 Time frame of the dissolution experiments, sampling and sample preparation

Sampling time points. The quick test (Part A) was developed to determine the apparent solubility of a NM under standard conditions (100 mg/L MN concentration, pH 7, 10 mmol/L $NaNO_3$ as background electrolyte) in a short timeframe and with minimal experimental efforts. It was decided to set two sampling points for this test, one immediately after preparing the test dispersion and the second after 24h. This can be easily performed in a commercial laboratory. Although the dissolution might not reach the solubility limit with less soluble NMs in that time span or might even over-saturate (Schmidt and Vogelsberger (2009)) still a useful value to assess the potential dissolution in environmental aquatic media will be retrieved. Because of the limited time-resolution a dissolution rate can only be roughly estimated from these two values. This test determines the apparent solubility of a NM after 24h (C_{s24h}).

Figure 4: Batch sampling and sources of error



Source: own figure, University of Vienna; RSD = relative standard deviation

The extended test (Part B) was developed to give a) access to the dissolution rate of the NM and b) the solubility after a period of 96h, at which an equilibrium between the solid and the solute is expected (Schmidt and Vogelsberger (2009)) and the solubility limit reached. It must be noted that a solubility in terms of the solubility limit of a NM is only correct if the NM has not fully dissolved. In all cases where the solid NM has fully dissolved, the solubility is larger than the initial concentration used. Taking into account that the dissolution will progress most dynamically during the first hours and a 8-hour working day and preparations required before the test can start, the sampling times have been set to immediately after preparation of the dispersions (0h), after 30 minutes (0.5h), 1h, 2h and 4h for the first day and then after 24h, 48h and 96h. This schedule delivers five datapoints in the early phase of dissolution what enables the determination of the dissolution rate for MNs which are not readily soluble. However, if a NM reaches its equilibrium solute concentration or dissolves completely within ≤4h its lifetime in the aqueous environment would anyway be extremely short.

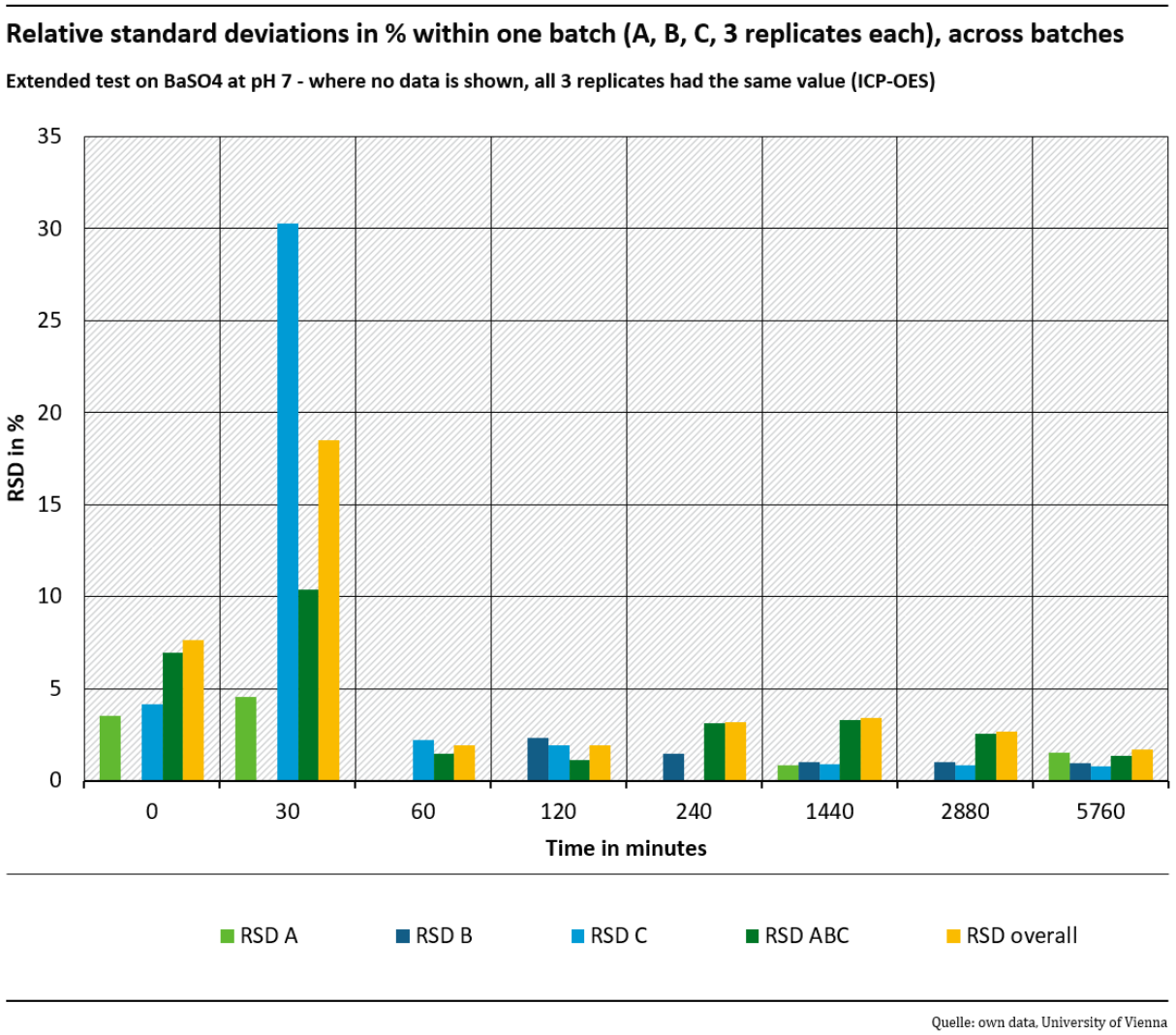
In Figure 4 the dissolution behavior of Ag-NPs is shown, and it is clearly visible, that silver nanoparticles continuously dissolve without reaching a plateau and equilibrium within 96 h.

This is caused by the fact that the metallic silver must first be oxidized before it can go into solution as a silver ion. This transformation-dissolution process is complex and leads to a limited but constant release (Molleman & Hiemstra 2017). It is not the scope of the developed dissolution tests to cover those complex coupled reactions which are influenced by many factors. However, if such a NM behavior is observed in the extended test and a concern, the duration of the test can be extended. In the case shown in Figure 4 the solubility of the formed Ag_2O (and other silver oxides) is higher than 1 mg/L and the NM in the test would have dissolved completely.

In Part C the sampling time points have been chosen by considering the volume required for analyses, the standard fraction collectors and the suggested flowrate of 1 ml/min. Sampling vials are typically 5 to 15 mL and as with Part A and B, 5 mL are a suitable sample volume considering expected concentrations, dilution factors and the analysis by ICP-OES or -MS. With these boundary conditions every 5 minutes a sample of 5 mL is taken. The sampling vials are pre-filled with 100 µL of 6 M nitric acid for the stabilization of the analytes. Some vials are kept without the addition of nitric acid to enable a pH measurement.

Sampling and ultrafiltration. In Part A and B 5 mL samples are taken from the agitated batches at the specified time points and immediately filtered by centrifugal ultrafiltration. As seen in Figure 4, the batch-to-batch error is far greater than the error between replicate filtrations. It has therefore been decided to perform the batch dissolution testing in triplicate batches and also the filtration is done in triplicates. The latter seems counterintuitive given the small variations between the filtrations. Outliers occur however and with single or duplicate filtrations this datapoint could be inaccurate or lost at all. Figure 5 shows the effect of having one outlier at timepoint 30 minutes (batch C reported 1.44, 0.68 and 1.44 mg/L Ba^{2+}) with the individual relative standard deviation (RSD) rising to more than 30% in batch C. By removing this one outlier almost all batches stay below 5%. The batch-to-batch variations at timepoint 0 are above 5% because of the low concentrations encountered at this early sampling point. Missing bars indicate a relative standard deviation of 0 % because all three replicates had the same value. Considering all influencing factors the repeatability is excellent.

Figure 5: Extended test (part B) barium sulphate at pH7 – relative standard deviations



Quelle: own data, University of Vienna

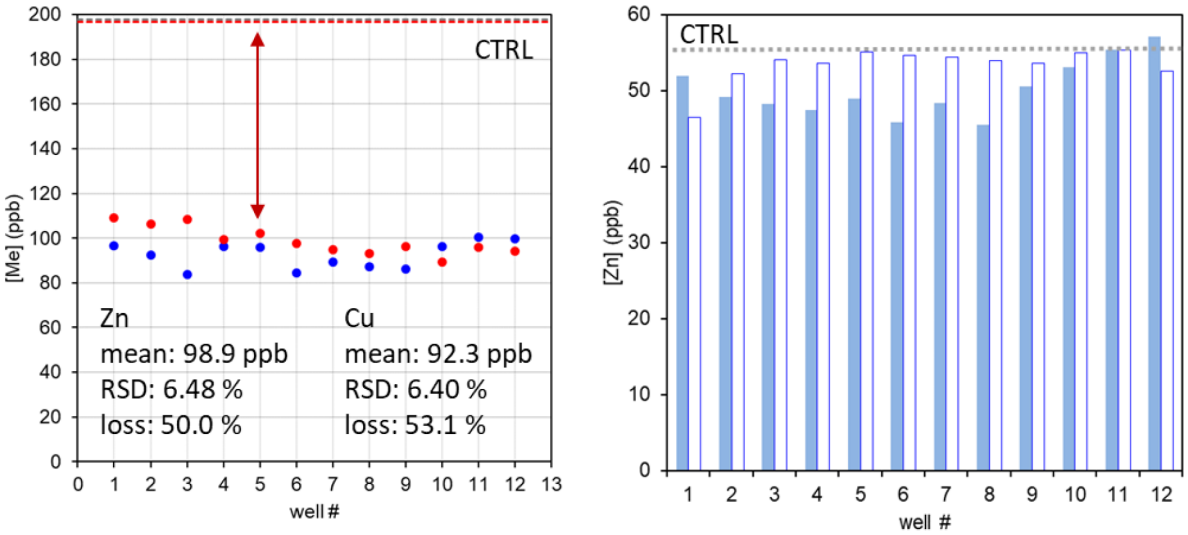
Centrifugal ultrafiltration and membrane pre-treatment. The finally adopted ultrafiltration strategy is a compromise between a full removal of the smallest possible NMs and a fast filtration. The fast filtration helps to determine the dissolution rate in the extended test, with the first time-point as close as possible to time zero and reducing the "blurring" of the result. Since the dissolution goes on in the dispersion during filtration and does this in an uncontrolled way, the exact time-point when the reaction reached the concentration measured in the filtrate is blurred. With shorter filtration times, this effect is reduced.

Usually ultrafilters of 1, 3 or 5 kDa MWCO are used to remove NMs from a dispersion. Here we decided for 10 kDa ultrafiltration units because the nominal pore size roughly only doubles from 1 kDa to 10 kDa and NMs larger than 3 nm should still be fully removed. A bovine serum albumin molecule has a molecular weight of 66.5 kDa and a Stokes diameter of ~ 7 nm. Since the exact time is less important in the quick test (Part A), here also ultrafiltration units with less than 10 kDa might be employed.

With 6 mL 10 kDa centrifugal ultrafiltration units spun at ≥ 3000 g in a standard table-top centrifuge the filtration is complete in ≤ 15 minutes while the ultrafiltration with 5 kDa units takes at least 30 minutes.

The ultrafiltration membranes in the centrifugal ultrafiltration units are known to adsorb small amounts of metal cations. When working with µg/L concentrations this can have a large effect on the accuracy of the filtrate analyses (Graef et al. 2023). Pre-treatment with another metal cation that adsorbs well to the membranes can reduce this effect. Figure 6 shows the losses of Cu and Zn on 3 kDa and 10 kDa units with and without pre-treatment.

Figure 6: Losses of analyte on ultrafiltration membranes before and after treatment



Losses of Zn and Cu during filtration of a nominal 200 µg/L solution (left). Losses to the 3k (filled bars) and 10k (open bars) membranes of a nominal 55 µg/L Zn solution after pre-treatment of the ultrafiltration unit with 500 µg/L $CuNO_3$ solution (right).

Source: own illustration; Intermediate Report 2022; University of Vienna

The losses on the tested units and without pre-conditioning can reach 100 µg/L in total and Greaf et al. 2023 reported even greater losses. With pre-treatment the losses are substantially smaller and 10 kDa membranes show smaller losses than 3 kDa membranes what is most likely due to membranes with lower MWCO have thicker membranes and more material available for sorption of the cations.

The finally adopted method for pre-treatment consists in the introduction of 5 ml of a 100 ppm copper nitrate ($CuNO_3$) or silver nitrate ($AgNO_3$) (if copper is among the target elements) solution. The tubes are then centrifuged at min. 3000 g for 15 min. The filtrated solution is discarded. Then, 5 ml of the medium is introduced into the tubes for the rinsing procedure and the tubes are centrifuged again at min. 3000 g for 15 min. The filtrated solution is again discarded. The bottom parts (receiver tube) should be dried with paper tissue or compressed air. The tube´s membrane itself must not be left to dry before filtration of the samples, this would lead to membrane blocking.

3.2 Part A – Batch tests for NM solubility using centrifugal ultrafiltration units (quick test)

The use of centrifugal ultrafiltration units is based on the following considerations:

1. Centrifugal ultrafiltration units (CUU) are available in various volumes, MWCO and membrane types at moderate costs.

2. CUU has become a standard routine technique, and the required equipment (table-top centrifuges with swing-out rotor) is available in all contract laboratories.
3. The units are easy to handle, with low risk of contamination or losses of the analyte.
4. The filtration in CUUs requires less time than the ultrafiltration with a vacuum filtration unit.
5. Standard vacuum filtration is costly (the membranes alone have a similar price-tag than the CUUs and it is not easy to multiplex (handling several samples at the same time)).
6. Ultracentrifugation is expensive, not always available, time consuming and the quality of the solid/liquid separation is dependent on the NM density and particle shape which then also determines the run conditions (g-force and time).

With respect to the volume of sample required for later analyses, the medium-sized 5 or 6 mL CUUs were chosen, which are based on the form factor of standard 15 mL Falcon tubes.

The test on solubility is performed at neutral pH (pH 7.00 ± 0.1) for 24 hours. It delivers only an apparent solubility, since the dissolution reaction might not have reached equilibrium after 24h. It is an operational value and a compromise between time requirements and reaching a stable equilibrium. Since equilibrium might be reached only after > 500 h a discussion about a test duration of 24, 48 or 96 h is futile, however, oversaturation < 70 h has been observed by Schmidt and Vogelsberger (2009) and should be considered. The experimental duration is subject to discussion within the OECD JEG.

The experimental set-up is to weigh 10 ± 1 mg of the test NM into an anti-static weighing boat, pre-wet with 1 mL of ethanol for 5 minutes and transfer with medium into acid-precleaned 125 mL bottles. PTFE or PFA bottles are preferred, because of the relatively high amount and initial concentration of the test NM also PMP or HDPE are suitable. The weighed amount must be reported with 0.1 mg precision. Then medium is filled into the bottles to a final volume of 100 mL. Comments from participants of the interlaboratory comparison suggest to use 250 mL bottles and 20 mg of NM in 200 mL as test volume. Reasons were the easier handling of larger NM amounts and more volume available in case of more replicates or a required repetition. Three parallel batches are prepared (triplicate batches).

The bottles are then vortexed for 1 minute.

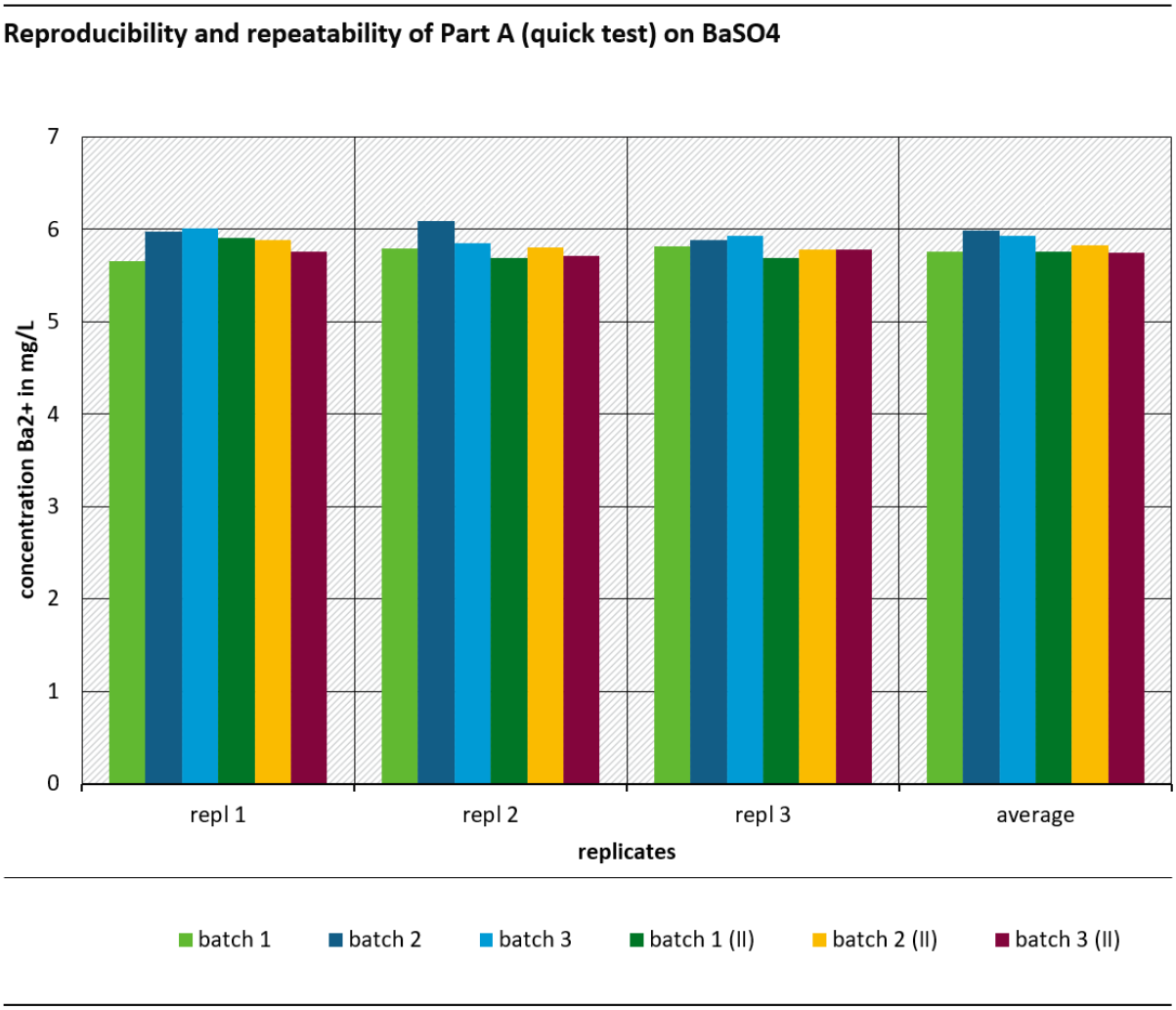
After this, the first samples of 5.00 mL are taken in triplicate from each batch (9 samples) and one blank medium. They are immediately centrifuged. While the centrifugation is underway, the pH in the three batches is determined and re-adjusted to 7.00 ± 0.1. An ion-meter with three digits resolution (7.000) is recommended, since the dissolution of many NM will be strongly pH dependent. The pH must be reported to assess the quality of the test and the correctness of the result.

The batches are placed lying flat on a horizontal shaker.

After 24 h the final set of triplicate samples are taken from the batches and prepared in the same way as the first set.

Figure 7 shows the results from two quick tests on barium sulfate. While the result is higher than the literature value of $BaSO_4$ solubility, the reproducibility (between replicates in the same test) and repeatability (between two tests conducted a month apart) is excellent.

Figure 7: In-house validation of Part A on barium sulfate



Source: own data, own graph; University of Vienna

While the 24-hour value represents the apparent solubility of the material, the initial (0 h) result shows the immediate dissolution during the sample preparation (for powders) and the pre-existing dissolved concentration of the NM in a dispersion. If dispersions are subjected to the test, they are diluted to the initial concentration of 100 mg/L if the original concentration of the NM dispersion is > 400 mg/L. If the concentration is < 400 mg/L a dilution of 1 to 4 is performed. This is done to dilute possible stabilizing agents and reduce the ionic strength which could have an effect on the solubility.

The tested NM is also digested and analysed to identify the purity of the NM with respect to the target element(s).

3.3 Part B – Batch extended tests for NM solubility and dissolution rate using centrifugal ultrafiltration devices

The developments focused on two different approaches. First the use of multi-well (centrifugal) ultrafiltration devices was tested for applicability. Second the method of Part A was applied in time intervals up to 168 h. The extended time interval > 96 h did not reveal any changes in solute concentrations for all test NMs but silver-NPs, for reasons mentioned earlier (p. 26), and 96 h were chosen as the maximum duration.

The 96-multi-well approach is easy, quick to operate and cost-effective (about 40% less per filtration than the single tube filtration). However, there are critical limitations:

1. Only 1 mL of sample volume is retrieved, posing some challenge for the analytics, especially when more than one element is the target analyte.
2. Special inserts for the centrifuge needed.
3. Special 8-channel pipettes needed.
4. Centrifugation must be complete, and this requires at least 60 minutes, blurring the initial phase of the dissolution process.
5. To gain full advantage of the concept, a special autosampler is required at the ICP-MS or ICP-OES to automatically and directly sample from the well-plate.

After exploring this concept in detail, it was given up in favour for the time-resolved variant of the Part A quick test which offers commonality between both test variants in terms of equipment and experimental approach.

The final design of the Part B extended test consists of:

1. Three pH values: 5, 7 and 8 to represent the range of pH encountered in natural waters.
2. Media composition as in Part A, but MES buffer applied for pH 5.
3. 200 mL as a batch volume with 2.0 ± 0.2 mg of NM (could be increased to 500 mL/5 mg for easier handling)
4. Number of replicates as in Part A.
5. 10 mg/L as initial NM concentration (will be discussed with the JEG).
6. Eight time-points for sampling: 0 h, 0.5 h, 1 h, 2 h, 4 h, 24 h, 48 h, 96 h to gain a good resolution in the beginning, to cover the time until an equilibrium (or full dissolution) should be reached and to respect a typical 8 h working day in a commercial laboratory

The buffers added to the media perform well over the extended period of the test. Two processes have an influence on the test pH: the consumption or production of protons by the NM's dissolution and the absorption of atmospheric CO_2 which tends to decrease the pH in the pH 7 and 8 tests. While the pH is kept constant in almost all tests (table 4), deviating max. ± 0.014 units from the average, the test of ZnO at pH 5 has a systematic deviation of up to + 0.17 units. This is due to the fact, that at the given amount of buffer in the media and its operational range, the instantaneous and complete dissolution of the 10 mg/L ZnO at pH 5 and the related consumption of protons cannot be fully countered by the MES buffer. This, however, will be reproducible and does not change the outcome in this case, since the ZnO dissolves completely during the test. For NMs with a solubility changing significantly between pH 4 and 6 this +0.17pH change may have a clear influence of the outcome. Therefore, the precise monitoring of the pH is mandatory to enable a correct evaluation of the test.

Table 4: Stability of pH during the extended test. $BaSO_4$ does not influence the pH during dissolution, ZnO consumes protons during dissolution and has a strong effect on pH

Time in minutes	pH 5 $BaSO_4$	pH 5 ZnO	pH 7 $BaSO_4$	pH 7 ZnO	pH 8 $BaSO_4$	pH 8 ZnO
0	5.018	5.156	7.000	7.057	7.995	8.036
60	5.016	5.168	7.001	7.061	7.985	8.037
120	5.018	5.170	6.996	7.063	7.983	8.039
240	4.993	5.155	6.981	7.052	7.975	8.029
1440	5.000	5.135	6.983	7.042	7.974	8.020
2880	5.002	5.152	6.984	7.050	7.972	8.016
5760	5.027	5.161	7.008	7.051	7.981	8.000

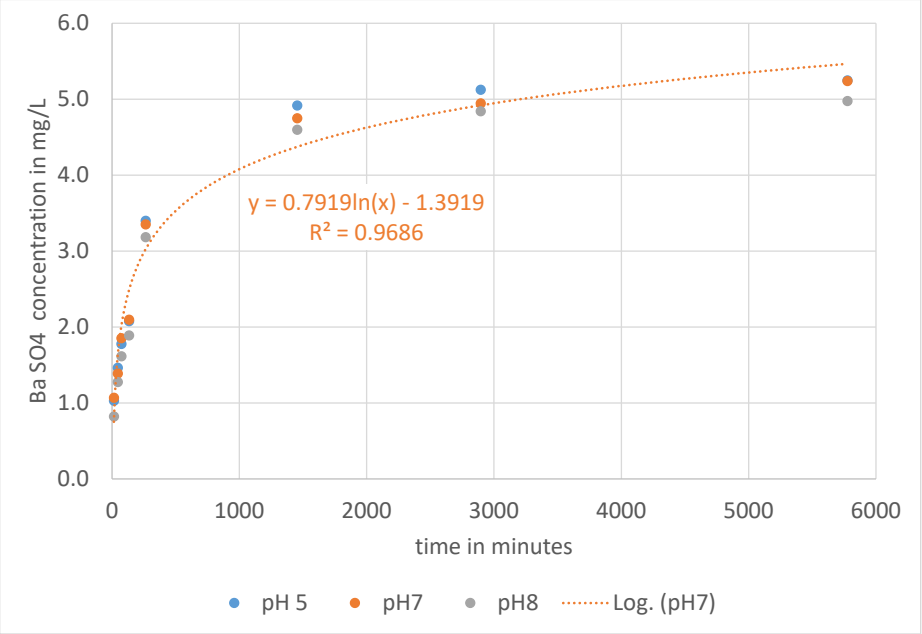
Figure 8 shows the dissolution behaviour of $BaSO_4$ at the different pH settings. Each datapoint is the average of triplicate filtrations from 3 batches (9 individual measurements). Even though the dissolution of $BaSO_4$ is independent of pH, the graph suggests a slightly higher solubility at lower pH. The dissolution starts at a high rate and then slows down after 4 hours approaching equilibrium after 2 days. The shape resembles a first order kinetic which can be fitted with a logarithmic function. The first derivative of this fit delivers the dissolution rate at any timepoint. Several possible deviations from the theoretical first order kinetic needs to be considered:

1. NMs usually show a distribution of particle sizes, the smaller ones will dissolve faster than the larger ones due to a larger specific surface area or (very small particles) showing a higher solubility.
2. The dissolution of the NM will continuously decrease the surface area of the NM.
3. Aggregates or agglomerates will dissolve slower since the dissolved ions need to diffuse through the pore space before they can dilute into the medium.
4. The sheer force of the medium at the surface of the particles (by shaking or stirring) regulates the thickness of the stationary layer. Those shear forces are less within an aggregate or agglomerate, slowing down the dissolution.
5. The structure (fractal dimension) and size of the agglomerate will also have an effect.
6. Crystallinity will have an effect, amorphous phases (e.g. on the surface of the particles) will dissolve faster than crystalline phases.
7. Different crystal planes might show different dissolution rates, the overall surface area is not equally contributing to the dissolution rate.
8. Particle shape as e.g. rods versus spheres will show different dissolution behaviour.
9. Complex NMs (e.g. mixed oxides) may dissolve non-stoichiometrically, different components will have different rates or the dissolution of one component gradually speeds-up the dissolution of another.
10. Coatings or layered structures will prevent an ideal dissolution behaviour.

It is therefore unlikely that the dissolution of a NM always follows the ideal first order kinetics. Very well soluble NMs may dissolve so quickly that fitting of a standard function might lead to erroneous results. ZnO e.g. reaches full dissolution within the first 30 minutes in pH 5 and 7. These considerations makes it difficult to suggest a standard procedure to extract dissolution

rates from the experimental results, which then leads to the need of individual interpretation of the data retrieved from the dissolution rate experiments of Part B – extended test.

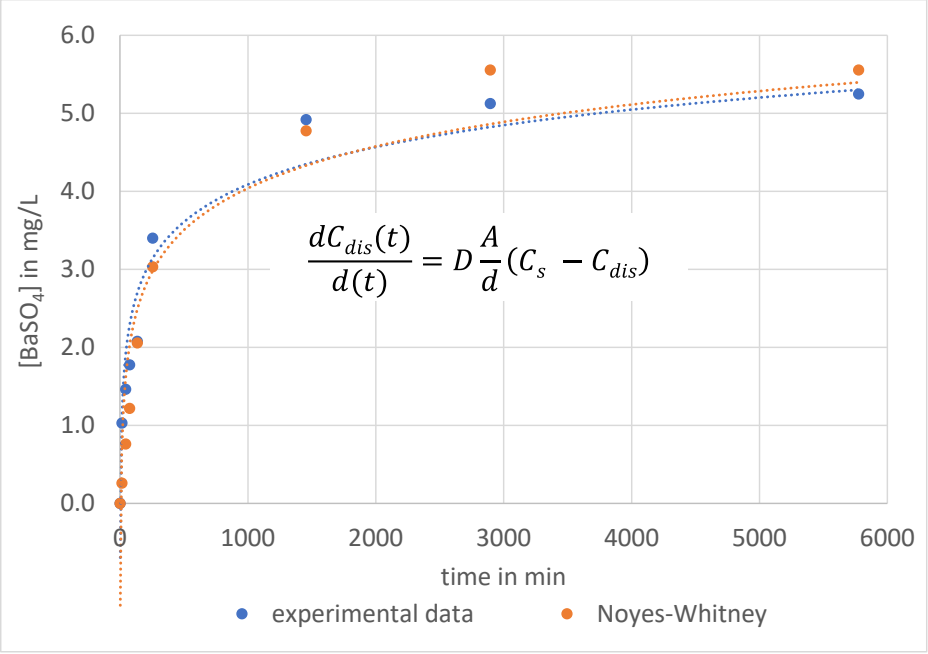
Figure 8: Time-dependent dissolution of $BaSO_4$ at different pH, tested with the methodology of Part B, pH 7 has been fitted with a logarithmic function, representing the first-order dissolution kinetics



Source: own data, own graph; University of Vienna

A solution would be to fit a logarithmic function to the part of the results, where the fit of the datapoints appears to represent the reaction well. This could require the restriction of the fitting to a certain time window.

Figure 9: Comparison of the experimental data with results from the Noyes-Whitney equation for the dissolution of $BaSO_4$ at pH 5. Parameters in the text.



Source: own data, own graph; University of Vienna

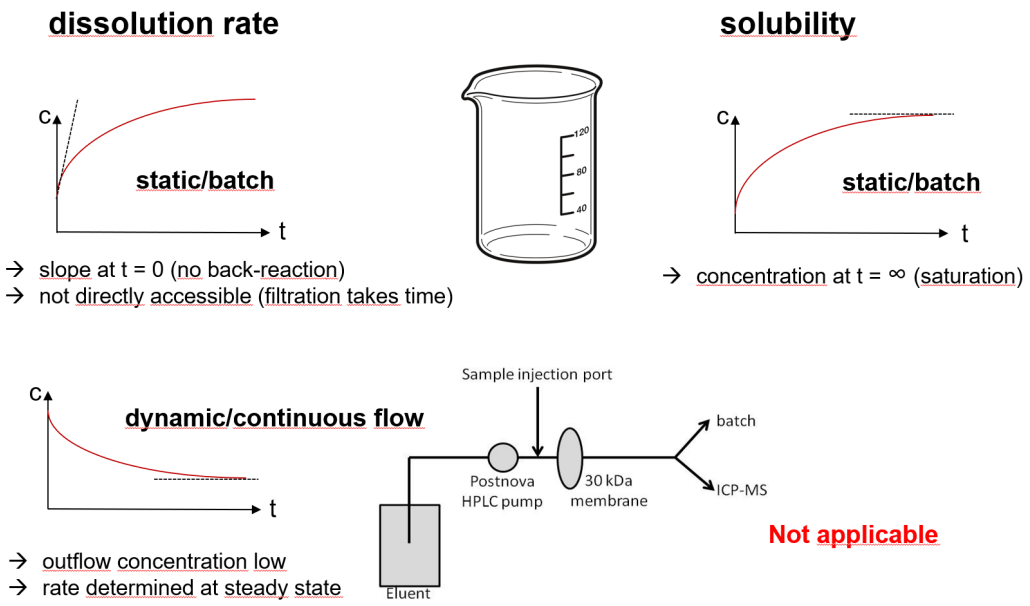
When comparing the obtained fit for the $BaSO_4$ tests at pH 5 with the results from the Noyes-Whitney equation, using the specific surface area *A* of the $BaSO_4$ of 40 m^2 (OECD NM 220) and a diffusion coefficient *D* for Ba^{2+} of 0.848 $*10^{-9}$ m^2/s we achieve a good overlap of both functions when assuming a thickness of the stationary layer of 65 nm, what cannot be measured but is a reasonable value under the given conditions (Figure 9).

From the fitting of the experimental values at t_{60min} we obtain an apparent dissolution rate of 0.48 $\mu g/m^2*sec$. The way how the experimental data should be treated, and which strategy should be implemented to extract the apparent dissolution rate from results of Part B – extended test is subject to discussions and decisions of the JEG.

3.4 Part C – Continuous flow dissolution tests to determine the NM dissolution rate

As an alignment to the testing strategies in OECD *TG 105 Water Solubility* and to enable a more realistic testing regime in which the NM is dissolving into a medium with vanishingly low concentrations of the solutes (infinite sink), a continuous-flow test has been adapted to represent relevant environmental conditions (Figure 3). The medium is continuously pumped through a filter holder in which the MN is placed on an ultrafiltration membrane. At the outflow an automated fraction collector collects a sample every few minutes and allows for a time-resolved analysis of the dissolution. The idea behind this approach is, that dissolved ions from the NM are immediately transported away and the NM dissolved into a medium from which a back-reaction is unlikely. With this the situation of a high liquid to solid ratio, comparable to the introduction of small amounts of NM into a large water body would be simulated.

Figure 10: Comparison of batch and continuous flow systems



Source: own graph; University of Vienna

The test ideally reports the maximum dissolution rate of the NM in an environmental setting. A similar test has been developed by Koltermann-Juelly et al. (2018) for conditions in the human lung. In this project, the test is operated at much higher flow rates and with different media. Figure 10 shows the principal differences of the batch/continuous flow approaches and how solubility and dissolution rate are determined.

The NM load on the membrane was initially kept to a minimum, with max. 10% of the membrane covered by the NM, providing an ideal single layer of NPs. At the chosen flow rates of 0.5 to 1 ml/min. a contact-time in the lower millisecond-range could be achieved. To enable this approach, it is necessary to bring the NM into a stable dispersion and inject small volumes of a dilute dispersion into the media stream before the filter-holder. Proof-of-principle with 25 mm filter holders and injection of small amounts of NM are reported in Stetten et al. 2022. Due to the fact, that only stable dispersions can be tested and the test showed an extraordinary long lag-time until stable steady-state conditions were reached, the experimental layout was changed and oriented at the one published by Koltermann-Juelly et al. (2018) with now 10 mg of NM placed in the filter holder and using filter holders made from PTFE to reduce possible contamination, losses and carry-over (Part C1). The flow rate has been set to 1 ml/min. Electrostatic charge is often encountered when loading the filter with NM and makes the weighing and even distribution on the filter membrane difficult. This can be countered by anti-static measures e.g. the ZeroStat3 anti-static pistol (Sigma Aldrich). Fan-based anti-static blowers bear the risk of NM losses and should be avoided. The duration of the test was set to 5 hours with a 5-minutes sampling interval. Every fourth sample is used to determine the evolution of the pH during the test.

To eliminate the need for an automatic fraction collector, an integrating continuous-flow test has been developed (Part C2), where the outflow of the system is collected in one single bottle, with a few intermediate sub-samples taken manually for pH control. At first sight this may appear similar to a static test with the final volume of 300 mL and 10 mg of NM in a batch. However, the fact that the solutes are not (or only fractions of a second) in contact with the NM provides a constant steep concentration gradient from the NM into the solution what accelerates the dissolution compared to a batch test. The obtained average dissolution over 5 hours is of course only a compromise. Problems might occur when the NM is well soluble and the 10 mg are quickly depleted.

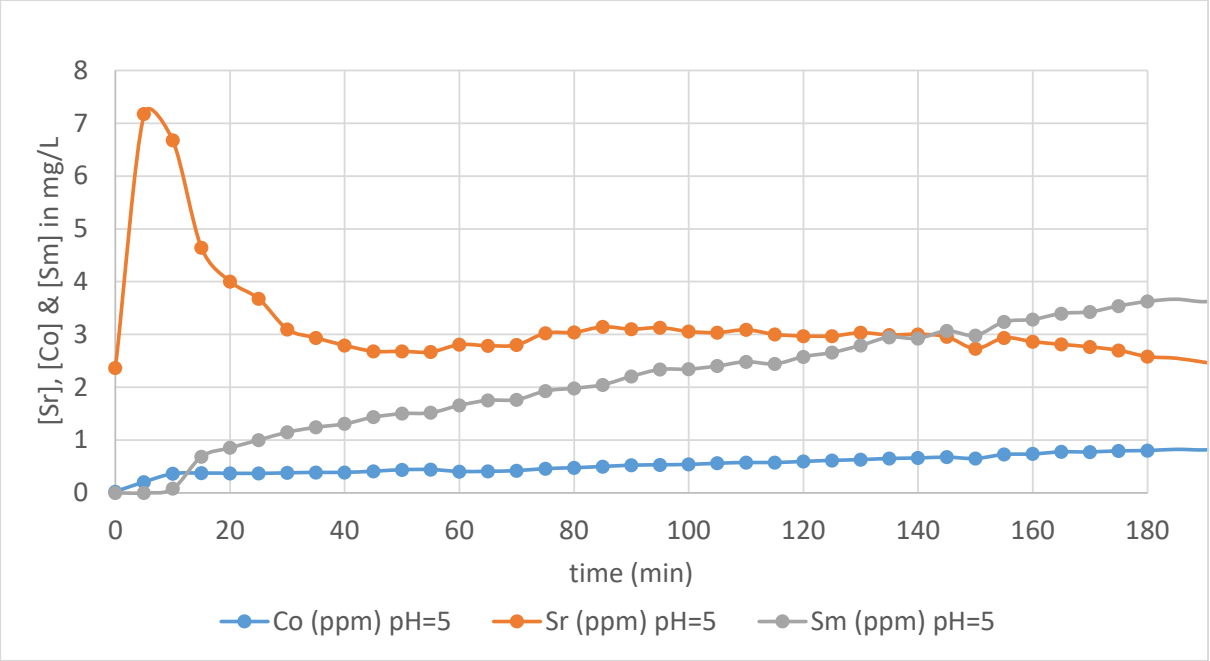
Overall, the results of the tests from the interlaboratory comparison show that the C1 test is well suited to determine dissolution rates when experienced personal is performing the test. In settings where the personal is performing the test for the first time some critical issues have been observed:

1. Outflow concentrations are continuously rising over the test: most likely due to air pockets in the filter holder which were removed over time. With this the fraction of NM in contact with the medium increases over time, leading to higher outflow concentrations.
2. Abrupt (within 30 minutes) changes in the outflow concentrations: unequal distribution of the NM on the filter or membrane breakage.
3. Not expected behaviour of the outlet concentrations, observed with the mixed oxide NM. This was reproducibly observed by more than one laboratory.

The last point must not be seen as a problem, the mixed oxide dissolves non-stoichiometrically and the dissolution of one metal seems to accelerate the dissolution of another. This can be nicely seen by the time-resolved experiment (Part C1, Figure 11) and adds valuable information

over a static batch test, but hinders the assessment of a dissolution rate for (in this case) samarium.

Figure 11: Dissolution profile of the mixed oxide over 180 min at pH 5



Source: own data; University of Vienna

3.5 Development of the SOP for the validation study

To validate the different part of the planned test guideline a standard operation procedure (SOP) has been developed and laboratories have been invited to participate in a round robin (interlaboratory comparison, ILC). The SOP has been extensively discussed with the OECD JEG on 8th of March 2023. An on-site training for participating laboratories at the Fraunhofer Institute IME in Schmallenberg took place on the 12th of September 2023 and two on-line question & answer sessions were provided to the participants on 12th of July and 9th of August 2023. The final SOP was sent out to the participants in January 2024, after issues with pH stability had been eventually solved. Data reporting sheets were provided to the participants and a data evaluation sheet to extract dissolution rates from Part B and C experiments is in preparation. 17 laboratories signed up to support the ILC, only two laboratories performed all tests from Part A to Part C2 on all NM provided.

The NMs selected for the ILC were $BaSO_4$ (NM-220), ZnO (NM-110) and a mixed oxide $Sm_{0.5}Sr_{0.5}Co_2O_x$. The reasons for the selection were:

1. **ZnO**: high pH dependency of solubility in the critical pH-range (5 to 8), dissolution increases pH, check for the stability of buffer systems and precision of pH-adjustment in the media
2. **$BaSO_4$**: medium solubility, no pH dependence; reveals possible errors other than pH instability; digestion procedure not straight forward
3. **Mixed Oxide**: fractional dissolution, non-stoichiometrically, pH dependence of solubility, dissolution increases the pH, 3 metals dissolve at different concentration levels (mg/L versus µg/L)

3.5.1 Preliminary results from the validation study

At the time of writing this report (October 2024), eight laboratories have reported results, and the evaluation of the results is ongoing. Figures 5 and 7 show the in-house results of the Part A and Part B on $BaSO_4$, which are similar for the other test materials. Single outliers can be observed in the triplicates of filtrations and be removed by outlier analysis. The 3 x 3 approach with 3 batches and 3 filtrations per batch and sampling point seems to provide a good basis for retrieving reliable data with ~ 5% of relative standard deviation (or even better). Dissolution kinetics could be retrieved from all $BaSO_4$ experiments, for pH 8 on ZnO and most of the metals in the mixed oxide at pH 7 and 8. The other experiments showed a too-fast dissolution which could not be assessed by the time-resolution of Part B.

To summarize, the Part A shows good reproducibility for $BaSO_4$ and less good, but acceptable, for ZnO. Part B has only been performed by two laboratories, however, since Part A is the basis of the Part B time series, we are confident that also Part B is reproducible. Problems occurred when e.g. the pH in the batches was not determined and controlled before and during the test.

Part C1 and C2 still needs further evaluation which will take place after the time of report preparation. One laboratory supplied excellent results.

Based on the received results of the ILC a validation report will be created. The observations and conclusions from the validation study will be used to develop a first draft of the test guideline. Both validation report and draft test guideline will be discussed with and commented by the OECD JEG on environmental fate and ecotoxicity testing starting from beginning of 2025. A new version of the draft test guideline based on the JEG feedback will then enter the OECD WNT commenting rounds starting in late summer 2025. It is aimed to submit the draft test guideline for adoption by OECD WNT at their 38th meeting in April 2026.

4 Conclusions

A universally applicable test system to assess the apparent 24-hour solubility and dissolution rate for NMs under relevant environmental conditions has been successfully developed. The test shows good repeatability and from preliminary ILC data also acceptable reproducibility. Precise control of pH (or lack of) seems to be the dominating factor for the variations observed.

In the current state the tests are restricted to metal- or metalloid-containing NMs, accessible by ICP-OES or -MS, but could be expanded to sulphur-, phosphor- and carbon-containing NMs when suitable analytical methods are available.

The Part B test allows for the determination of dissolution kinetics for medium to low soluble NMs. From tests with BaSO4 and ZnO @ pH 8 a rate determination should be possible for NMs with less than ~ 5 mg/L solubility.

The Part C tests delivered a mixed picture from the ILC with usable results coming from very experienced laboratories and some outliers from less experienced.

The SOP developed and testing within the ILC will now be further elaborated and discussed at OECD WNT level. Experts from the JEG will review the proposed protocols for PART A-C, also considering the outcomes of the ILC. This will be followed by official commenting rounds of the WNT with the objective to adopt these protocols as a new OECD TG on solubility and dissolution rate of NMs in aquatic media. This project provides the experimental basis for this TG.

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