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Review of methodologically problematic issues regarding the examination of nanomaterials in the environment – Development of a decision support tool for the investigation of environmental behavior of nanomaterials on the basis of their dispersion stability and dissolution as a function of environmental conditions

by
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Kurzbeschreibung

Der Projektschlussbericht beschreibt die Überlegungen, experimentellen Ansätze und Ergebnisse, die die Grundlage bilden für eine neue OECD Testrichtlinie zur Dispergierbarkeit und zum Dispersionsverhalten von Nanomaterialien in wässrigen Medien unterschiedlicher, naturrelevanter Zusammensetzung. Darüber hinaus wird das Ergebnis einer Literaturrecherche zu Transformationsreaktionen von Nanomaterialien in Umweltmedien (Stand 2015) dargestellt.

Grundlage der experimentellen Ansätze war das Dispersionsverhalten in möglichst wenigen und einfachen Tests unter für natürliche Bedingungen repräsentativen und gleichzeitig für das Dispersionsverhalten relevanten hydrochemischen Bedingungen untersuchen zu können. Nanomaterialien sollten so in ihrem Verhalten in standardisierten Verfahren untereinander verglichen werden können. Grundlage des experimentellen Ansatzes waren Vorarbeiten zum Dispersionsverhalten von Titandioxid-Nanopartikeln mit deren Hilfe eine sinnvolle Reduktion des Parametersatzes durchgeführt werden konnte. Die entwickelte experimentelle Methode basiert auf einem Homoagglomerations-Verfahren, in dem der Verlauf der Agglomeration durch Beprobung und Bestimmung der verbliebenen Partikelmassenkonzentration im obersten Zentimeter des Dispersionsüberstandes eines 50 ml Reaktionsgefäßes gemessen wird. Mit fortschreitender Agglomeration sedimentieren die Partikel und es werden zeitabhängig die Partikelmassenkonzentrationen im Überstand gemessen. Die Startkonzentration ist als Anzahlkonzentration definiert und in einer Spanne von einer Größenordnung festgelegt. Die für die Erreichung der definierten Anzahlkonzentration einzusetzende Massenkonzentration wird auf Basis des mittleren Partikeldurchmessers und der Materialdichte errechnet. Die Zeitspanne des Tests wurde auf sechs Stunden festgelegt, was direkt mit der zu verwendenden Anzahlkonzentration am Testbeginn korrespondiert, da die Kinetik des Homoagglomerationsverhaltens von der Anzahlkonzentration am Beginn abhängt. Der Test wird mit einem Zentrifugationsschritt abgeschlossen, um die unterschiedliche Dichte der Partikel zu berücksichtigen.

Die hydrochemischen Bedingungen wurden so gewählt, dass rund 95% der in der Natur vorkommenden Gehalte an für die De-Stabilisierung der Partikel verantwortlichen Elektrolyt, gelöste organische Substanz-Konzentrationen, und pH-Werte berücksichtigt werden.

Es wurde ein Entscheidungsbaum entwickelt nach dem bestimmt wird, ob das Material einer einfachen oder eingehenderen Testung unterzogen werden muss und ob es am Ende als stabil dispergierbar, nicht dispergierbar bzw. abhängig von den hydrochemischen Bedingungen dispergierbar ist.

Als Materialien zur Überprüfung des Konzepts wurden Silber-Nanopartikel (NM300K), Titandioxid-Nanopartikel (NM105) und Kohlenstoff-Nanoröhren (NM400) verwendet.

Die Testrichtlinie, die basierend auf den hier vorgestellten Arbeiten entwickelt wurde, wurde dem OECD Council zu Verabschiedung vorgelegt und kann danach auf den Seiten des OECD Testrichtlinienprogramms abgerufen werden: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthe-testingofchemicals.htm

Abstract

The final report of this research project describes the considerations, experimental approaches and obtained results which build the basis for a new OECD test guideline for the dispersibility and dispersion behavior of nanomaterials in aqueous media of different, environmentally relevant hydrochemistry. The report also contains a literature review of transformation reactions of nanomaterials in environmental media (up to 2015).

Prerequisite for the experimental approach was to enable the investigation of the dispersion behavior in a small number of relatively simple tests. The hydro-chemical conditions in the tests should cover only those parameters and parameter ranges which are representative for natural waters and relevant

for the dispersion behavior of nanomaterials at the same time. The test should provide an operationally defined and standardized procedure to compare nanomaterials regarding their dispersion behavior. The basis for the experimental approaches were earlier systematic studies on the dispersion behavior of different titanium dioxide nanomaterials which helped to reduce the parameter set in a reasonable way. The developed test routine is based on homo-agglomeration where the progressing of agglomeration and settling is followed over a defined time-span by analyzing the remaining concentration of the nanomaterials in the supernatant of the dispersion. With progressing agglomeration the concentration in the supernatant will decrease over time. The starting concentration is standardized as a particle number concentration in the range of one order of magnitude. To obtain the required mass concentration the defined number concentration has to be converted using the average particle diameter and material density which is described in the new test guideline. The duration of the test was fixed to six hours and is directly linked to the starting particle number concentration. The kinetic of the homoagglomeration process is depending on the number concentration of the particles. The test is completed with a centrifugation step at the end to account for different densities of the nanomaterials.

The hydro-chemical conditions were chosen to represent about 95% of the conditions found in natural waters. As being relevant for the de-stabilization of nanomaterial dispersions the test considers the concentrations of important electrolytes (di-valent ions), natural dissolved organic matter and pH.

A decision tree has been developed which is used to determine if the material of interest requires only a screening procedure or if an in-depth testing has to be performed. The decision tree allows categorizing nanomaterials as generally stable dispersible, non-dispersible or dispersible depending on the hydro-chemical conditions.

For a proof-of-principle silver nanoparticles (NM300K), titanium dioxide nanoparticles (NM105) and carbon nanotubes (NM400) were employed.

The OECD TG developed based on the research presented in the report is intended for OECD council adoption and will then be available at the webpage on OECD Guidelines for the Testing of Chemicals: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

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Definitions, units and abbreviation

Ag NP	silver nanoparticles (specific nanoform NM300K used in this study)
Agglomeration	Reversible clustering of initial small particles in space, happening due to interparticle interactions through various attraction forces and resulting in formation of larger secondary particles (agglomerates)
Agglomeration behavior (Dispersion stability)	Shows the change of particle concentration in the top 1 cm of dispersion over and during time period of 6 hours. Thus the term “dispersion stability” includes the term “dispersibility” and is recommended for further use.
CNT	carbon nanotubes (specific nanoform NM400 used in this study)
Concentrations	Concentrations of the particles and DOM in the samples are given in g/L, mg/L (ppm) and µg/L (ppb). Concentrations of salts ($Ca(NO_3)_2$ and $NaHCO_3$) present in the samples are given in mM
Dispersibility	Particles should be considered dispersible if after 6 hours of experimental time the concentration of particles in the top 1 cm of dispersion exceeds or equals 10% of initial particle concentration at the initial observation time (t0)
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EDL	Electric Double Layer
ENM	engineered nanomaterial, synonym to manufactured nanomaterial
ENP	engineered nanoparticle, synonym to manufactured nanoparticle
Experimental endpoint	Measurement that shows remaining concentration of particles in the top 1 cm of dispersion after 6 hours of experimental time, related to the initial concentration of particles, detected during first measurement, and expressed in percentage (%), such as: experimental endpoint = $(C6h/C0) \times 100$
Experimental time	Time, required to test the dispersibility of particles or their dispersion stability is measured in hours (h). The recommended experimental time is 6 hours
GD	Guidance Document
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy

Nanomaterial	Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale (ISO/TS 27687:2008).
Nanoparticle	Nano-object with all three external dimensions in the nanoscale. In this Report we also denote carbon nanotubes as nanoparticles, although they might fall out of this definition for their length not being in the nanoscale (ISO/TS 27687:2008).
NOM	Natural Organic Matter A general term for a substance resembling the natural organic matter in water and soils, if dissolved in water the soluble part becomes the DOM
NTU	Nephelometric Turbidity Units (nephelometry/ turbidity measurements)
PZC	Point of Zero Charge, such state at the surface of the material when there are no charged moieties (theoretical value). In contrast to PZC, IEP - Isoelectric Point is such a state on the material surface when the surface net charge equals to zero
RRT	Round Robin Test
RSD	Relative standard deviation
Size	Size of the particles is given in micrometers (µm) or nanometers (nm) and can be determined by using TEM imaging, or in (d.nm) using DLS size measurements, or provided by supplier information. Besides, unit measurement methods should be specified.
TABS	Tiered Agglomeration Behavior Scheme
TG	Test Guideline
TiO_2	Titanium dioxide (specific nanoform NM105 used in this study)
UV-VIS	Ultraviolet-visible spectroscopy
WNT	Working Group of the National Coordinators of the Test Guidelines Programme

Zusammenfassung

Im Laufe des OECD - WPMN Expert Meeting on Environmental Fate & Eco-Toxicology of Nanomaterials in Berlin im Januar 2013 (OECD 2014) entwickelte sich ein gemeinsamer Standpunkt bei den Experten, dass ein Bedarf für mindestens drei neue, nanomaterialspezifische Prüfrichtlinien zur Frage des Agglomerationsverhaltens, der Auflösung und der Transformation von Nanomaterialien existiert. Es wurde beschlossen zwei Prüfrichtlinien (Test Guidelines, TG) zusammen mit einem gemeinsamen Leitfaden (Guidance Gocument, GD) für die Dispersionsstabilität und das Auflösungsverhalten von Nanomaterialien zu entwickeln. Ziel war es, zwei OECD TGs zu entwickeln, die die experimentellen Routinen liefern, um das Agglomerations- und Auflösungsverhalten von Nanomaterialien unter den für diese Prozesse relevanten Bedingungen in natürlichen Wässern zu bestimmen. Die Ergebnisse der Prüfungen sollen es ermöglichen das Umweltverhalten der Materialien abzuschätzen, Aussagen darüber liefern welches Verhalten in den nachgeschalteten ökotoxikologischen Tests zu erwarten ist und beschreiben welche nanospezifischen Eigenschaften bezüglich Agglomeration und Auflösung in den Tests zu berücksichtigen sind. Ziel dieses Projektes war es, eine Prüfrichtlinie zur Dispersionsstabilität bzw. dem Agglomerationsverhalten von Nanomaterialien unter umweltrelevanten hydrochemischen Bedingungen zu entwerfen sowie erste Anhaltspunkte für einen Leitfaden zu dieser Prüfrichtlinie und der Prüfrichtlinie zum Auflösungsverhalten zu identifizieren (letztere nicht Teil dieses Projekts). Dieser Bericht enthält die experimentellen Ergebnisse, die während der Entwicklung der Methode zur Dispersionsstabilität in simulierten Umweltmedien erhalten wurden. Da Nanomaterialien in Testsystemen auch Umwandlungsreaktionen durchlaufen können, ist ein Teil dieses Berichts diesen Prozessen gewidmet. In Form einer Literaturstudie wird der Stand des Wissens in 2015 über typische Umwandlungsreaktionen dargestellt. Es ist anzumerken, dass der Wissensstand zu dieser Fragestellung seit damals schnell voran geschritten ist.

Technische Nanomaterialien (ENMs) verhalten sich in der Umwelt anders als gelöste Substanzen, Partikel › 1 µm oder Feststoffe. Durch ihre geringe Größe wird das Verhalten der Partikel weitgehend durch ihre Oberflächenchemie bestimmt und außer bei Partikeln mit hoher Dichte überwiegt in wässrigen Medien die Diffusion der Partikel ihre Sedimentation und die Partikel bleiben über längere Zeit im Schwebezustand (in Abhängigkeit von der Dichte sogar bis hin zu mikroskaligen Partikeln). Dies führt zur Ausbildung von Dispersionen selbst ohne zusätzliche Maßnahmen zur Stabilisierung. Eine Dispersion ist ein makroskopisch homogenes, flüssiges Medium in dem mikroskopische Phasenübergänge fest/flüssig existieren. Dispersionskolloide, zu denen viele ENPs gehören, sind thermodynamisch jedoch instabil, neigen dazu zu agglomerieren und sich durch Sedimentation der Agglomerate abzuscheiden. Dispersionskolloide können durch interpartikuläre Kräfte, die das Agglomerieren der Partikel verhindern, in einem pseudostabilen Zustand verbleiben. Das besondere Verhalten der ENPs lässt es naheliegend erscheinen, dass die Risikobewertung auf Verfahren zurückgreifen muss, die diese Besonderheiten gegenüber gelösten Substanzen oder klassischen Feststoffen berücksichtigen.

Die Ergebnisse aus Untersuchungen zur Dispersionstabilität und auch Auflösung lassen Rückschlüsse zu, ob und wenn ja, wie die Nanomaterialien in weiterführenden Prüfungen nano-spezifisch betrachtet werden müssen. So würde ein leicht und schnell lösliches Partikel u.U. klassisch wie lösliche Substanzen getestet werden, während für un-/teillösliche Partikel eine spezifische Prüfstrategie abgeleitet werden könnte. Beispielsweise würde ein unlösliches, gut dispergierbares Partikel eher zur Exposition von pelagialen Organismen führen, während bei schnell agglomerierenden, schlecht dispergierbaren Materialien Sedimentorganismen verstärkt exponiert würden. Dies könnte bei nachfolgenden Tests und deren Bewertung berücksichtigt werden.

Die Ergebnisse der einzelnen, hier dargestellten Experimente dienen der Gestaltung der OECD Testrichtlinie zur Testung des Dispersionsverhaltens in unterschiedlichen aquatischen Medien. Die Grundidee der dazu entwickelten Prüfmethode ist, eine Dispersion des zu testenden Nanomaterials

unterschiedlichen aber typischen hydrochemischen Bedingungen in einem Oberflächengewässer auszusetzen und die Stabilität der Dispersion über die Zeit zu beobachten. Dies soll über regelmäßige Probennahmen kurz unter der Oberfläche der Dispersion und Konzentrationsbestimmung der darin (noch) enthaltenden ENPs erfolgen. Bei einer stabilen Dispersion wird diese Konzentration konstant sein, andernfalls sie sich durch Agglomeration und Sedimentation der Partikel verringern.

Partikel kleiner als etwa 1 µm Durchmesser bilden thermodynamisch instabile Dispersionen in wässrigen Medien. Solche lyophoben Dispersionskolloide (im Unterschied zu lyophilen Mizellen oder Molekülkolloiden), zu denen die meisten der derzeit betrachteten Nanopartikel gehören, können durch interpartikuläre Abstoßungskräfte in Dispersion gehalten werden. Die dafür notwendige Energiebarriere wird hauptsächlich durch elektrostatische und sterische Wechselwirkungen erzeugt. Andere Wechselwirkungen spielen ebenfalls eine Rolle (siehe Grasso, et al. 2002), sollen hier aber nicht betrachtet werden. Diese Wechselwirkungen werden kontrolliert durch die Materialeigenschaften, die Oberflächenchemie und die Zusammensetzung des die Partikel umgebenen Mediums (Wasserchemie). Je nach Partikeleigenschaften und der umgebenden Wasserchemie können sich demnach relativ stabile Dispersionen ausbilden oder die Partikel agglomerieren und sedimentieren. Die Zusammensetzung von natürlichen Wässern ist sehr komplex und räumlich/zeitlich variabel. Um das Verhalten der Partikel in natürlichen Wässern oder Testmedien zu bestimmen, kann man eine Dispersion der Partikel in einem bestimmten natürlichen Wasser oder Medium herstellen und das Verhalten der Partikel beobachten. Die hieraus erhaltenen Informationen mögen sehr realistisch für das untersuchte Wasser-/Partikelsystem sein, sind aber nicht übertragbar auf andere Wässer/Medien und die das Verhalten der Partikel bestimmenden Eigenschaften des Wassers/Mediums können nur schlecht oder gar nicht in Erfahrung gebracht werden. Im Rahmen von vorausgehenden Untersuchungen (Liu, et al. 2013, Ottofuelling, et al. 2011, von der Kammer, et al. 2010) konnten einige wenige Parameter als Haupteinflussfaktoren isoliert werden. Hierzu wurden die in nicht-marinen Oberflächengewässern normalerweise anzutreffenden Konzentrationsbereiche unterschiedlicher Kationen und Anionen berücksichtigt und mit dem Ausmaß ihres Einflusses auf die Agglomeration kombiniert. Die resultierenden primären Einflussfaktoren sind danach der pH-Wert, die Calciumionenkonzentration und die Konzentration natürlicher organischer Substanz. In einigen Wässern und bei bestimmten Partikeln können auch Magnesium- und Sulfationen einen Beitrag zur Agglomeration zeigen, um solche Wässer ebenfalls abzubilden kann mit $MgSO_4$ als Elektrolyt gearbeitet werden; zusätzlich zu dem hier verwendeten $Ca(NO_3)_2$ und im häufig anzutreffenden Molverhältnis von 1:4 für Mg zu Ca. Die gewählten Salze ergeben sich aus der Betrachtung ihres Vorkommens in Gewässern und ihres Einflusses auf die Dispersionsstabilität hauptsächlich elektrostatisch stabilisierter ENMs: die einwertigen Kationen (Na, K) haben nur geringen Einfluss auf die Agglomerationsneigung stabiler Dispersionen, während die in ähnlich hohen Konzentrationen vorkommenden zweiwertigen Kationen (Ca, Mg) aufgrund ihres weit größeren Einflusses auf die Stabilität (Schultze-Hardy-Regel) den Prozess dominieren. Dreiwertige Kationen (Al, Fe) haben noch einen weit stärkeren Effekt, kommen aber nur in vernachlässigbaren Konzentrationen vor. Die für die Tests ausgewählten Konzentrations- und pH-Wertbereiche decken etwa 95% der Werte ab, die in Europäischen Oberflächengewässern gefunden werden (Salminen 2007) und daher einen weiten Bereich realer Konzentrationen. Während es praktisch unmöglich ist, die vollständige Bandbreite natürlicher Wässer im Labor zu testen, ermöglicht die für die OECD TG vorgeschlagene Prozedur eine systematische und vergleichsweise einfache Abschätzung auf Basis der wichtigen Parameter.

In natürlichen Gewässern wird die Konzentration ENMs um Größenordnungen unter denen natürlicher Partikel liegen. Daher ist die Agglomeration der ENMs mit natürlichen Partikeln (Heteroagglomeration) von größerer Bedeutung für den Transport und Verbleib, als die Homoaggregation zwischen den ENPs selbst. Dies gilt jedoch nicht unbedingt für die standardisierten OECD Prüfsysteme, in denen die ENMs die einzigen partikulären Feststoffe darstellen können. Es würde sich auch die Frage stellen, was ein standardisiertes Material sein könnte, das den Gegenpart in einem Test zur Heteroagglomeration darstellen könnte (Schwebstoffflocken, Klärschlamm, Sedimentpartikel?). Natürliche Partikel wie Gewässerschwebstoff sind sehr komplex und zeitlich und räumlich sehr variabel in ihrer Zusammensetzung und Gestalt. Darüber hinaus sind experimentelle und analytische Ansätze zur Messung der Heteroagglomeration noch nicht weit entwickelt. Erste Ansätze zu einer Bestimmung von Heteroagglomerationsprozessen in einem experimentellen Ansatz, der dem hier vorgestellten ähnelt, gibt es seit kurzem (Barton, et al. 2014) und das in diesem Projekt von Anfang an verfolgte Konzept eines sinnvoll vereinfachten Testverfahrens wurde kürzlich allgemein für die Testung von ENPs beschrieben (Hendren, et al. 2015). Das hier beschriebene Verfahren stellt eine gut begründete Vereinfachung von weit umfangreicheren Tests dar (Ottofuelling, et al. 2011) und verzichtet absichtlich auf die Einbeziehung von Heteroagglomerationsprozessen wegen der derzeit noch vorherrschenden Probleme bei der Definition eines dafür geeigneten Testsystems. Die Parameter, die für den hier entwickelten Test relevant sind und auf ihre Auswirkungen auf das Ergebnis untersucht wurden, sind:

Ionenstärke – kontrolliert die Ausdehnung der elektrochemischen Doppelschicht an der Oberfläche der Partikel (Debye-Länge κ^{-1}) und damit das Ausmaß und die Reichweite der elektrostatischen Wechselwirkungen zwischen den Partikeln. Die Ionenstärke berücksichtigt die Konzentration und die Valenz aller beteiligten Ionen einer Lösung. Die Valenz der zur Partikeloberflächenladung entgegen geladenen Ionen beeinflusst aber die Stabilität von elektrostatisch stabilisierten Dispersionen (z.B. die Kritische Koagulationskonzentration) deutlich stärker (Schulze-Hardy Regel) als dies durch den Anstieg der Ionenstärke bei Erhöhung der Valenz wiedergegeben wird. Im Test wird die Konzentration von $Ca(NO_3)_2$ (ergänzt alternativ um $MgSO_4$) zwischen 0 und 10 mM variiert.

pH-Wert – für Partikel mit variablen Oberflächenladungen (z.B. Metalloxide, amphotere organische Überzüge, Überzüge mit Carboxyl- oder Aminogruppen) spielt der pH-Wert eine große Rolle für die Ladung der Oberfläche und damit für die Stabilität einer wässrigen Dispersion dieser Partikel. Der pH-Wert kontrolliert dabei sowohl die Ladungsdichte auf der Oberfläche (durch Protonierung/ Deprotonierung) als auch die Polarität der Oberflächenladung bei amphoter reagierenden Oberflächen. Am Ladungsnullpunkt (point of zero charge PZC) fehlen die Abstoßenden Elektrostatischen Kräfte und die Dispersion ist instabil. Der PZC ist eine Materialeigenschaft und ist gleich dem isoelektrischen Punkt (isoelectric point IEP) wenn keine spezifische Adsorption von Bestandteilen des Mediums an der Oberfläche vorliegt. Im Test wurde der pH Wert zwischen 5 und 8.5 variiert.

Konzentration natürlicher organischer Substanz (dissolved organic matter DOM) – in vielen Fällen erhöht die Anwesenheit von DOM im Wasser/Medium die Stabilität einer Dispersion. Dies liegt hauptsächlich an drei Faktoren: zum einen adsorbiert die DOM an die Oberflächen vieler ENPs und erhöht dort die negative Ladung durch die im neutralen pH-Bereich de-protonierten Carboxylgruppen der DOM. Diese Erhöhung der negativen Ladung verbessert die Abstoßung zwischen den Partikeln. Die DOM Moleküle verhindern auch durch sterische Effekte eine Annährung von Partikeln und drittens komplexiert DOM viele mehrwertige Kationen wie Calcium sehr effektiv und reduziert damit die Aktivität des Kations und damit seinen Beitrag zur De-stabilisierung der Dispersion. Im Falle einer positiven Oberflächenladung würde die Zugabe von DOM die Ladung zunächst vom Betrag her reduzieren (bis zum IEP) und damit die Dispersion de-stabilisieren, eine weitere Zugabe würde dann eine negative Oberflächenladung etablieren und wieder zu Stabilität der Dispersion führen. Im Test wurden DOM Konzentrationen zwischen 0 und 30 ppm (als DOC, dissolved organic carbon) eingesetzt.

Partikelkonzentration – das Prinzip des Tests basiert auf der Agglomeration der Partikel und dem nachfolgenden Sedimentieren. Damit unterschiedliche Materialien miteinander verglichen werden können muss der Agglomerationsvorgang für ähnlich instabile Dispersionen auch ähnlich schnell verlaufen. Da die Partikel-Anzahlkonzentration ein entscheidender Faktor für die Geschwindigkeit des Prozesses ist, muss diese für unterschiedliche Materialien am Anfang möglichst gleich sein. Es wurde untersucht, wie genau diese Anfangskonzentration einzustellen ist.

Partikelgröße und -dichte – eine instabile Dispersion wird durch Agglomeration der ENPs und Sedimentation der gebildeten Agglomerate im Test erkannt. Die Sedimentationsrate wird hierbei durch die Größe der Aggregate und ihre Dichte bestimmt (zu einem Teil auch durch die Form und Durchströmbarkeit der Agglomerate, dies soll aber hier unberücksichtigt bleiben, da beide Eigenschaften schwer zu bestimmen sind). Gerade Agglomerate aus Materialien mit einer nur leicht höheren Dichte als die des Mediums können während der Dauer des Tests in Schwebe bleiben und so eine stabile Dispersion vortäuschen. Daher wird das Testgefäß am Ende des Tests zentrifugiert und über die Berechnung einer Zentrifugationszeit (Berechnungshilfe als Anhang zur Prüfrichtlinie), die eine einheitliche Partikelgröße (1 µm) und die Dichte des Materials berücksichtigt, wird wieder Vergleichbarkeit hergestellt.

Rolle des atmosphärischen CO_2 – wie zuvor bereits angeführt können Substanzen, die auf die Oberfläche der Partikel sorbieren, die Ladung der Oberfläche beeinflussen. Damit gilt dann IEP≠PZC. Da der IEP wichtig für die Dispersionsstabilität ist, wurde der Einfluss von CO_2 auf die Dispersionsstabilität untersucht. Es war davon auszugehen, dass aus CO_2 gebildetes und sorbierendes CO_3^{2-} den IEP zu niedrigen pH-Werten verschiebt. Auch wenn dieser Effekt in der Literatur häufig beschrieben ist, konnte keine Veränderung der Dispersionsstabilität der getesteten Materialien mit und ohne Luftabschluss gefunden werden.

Das Ergebnis des hier entwickelten Tests beschreibt die Dispergierbarkeit/Dispersionsstabilität sowie das Agglomerationsverhalten unter standardisierten Bedingungen.

Dispergierbarkeit – beschreibt wie gut ein partikuläres Material in einem Medium zu einer Dispersion verarbeitet werden kann. Viele Produkte unseres täglichen Bedarfs sind (meist recht stabile) Dispersionen oder Emulsionen. Das zeigt, dass viele partikuläre Materialien unter Zuhilfenahme von Hilfsstoffen und Energiezufuhr dispergierbar sind. Die Frage hier ist jedoch wie sich ENMs unter Bedingungen, wie sie in natürlichen Oberflächengewässern existieren, dispergieren lassen. Die Dispergierbarkeit ist also immer systembezogen und kann nur für eine bestimmte Prozedur angegeben werden. In der hier definierten Methode (in Anlehnung an existierende Protokolle) wird zunächst eine höher konzentrierte Vorratsdispersion produziert (definiert sind Massenkonzentration ENM, Ultraschallsonde und eingetragene Ultraschallenergie) aus der verdünnte Dispersionen unterschiedlichen hydrochemischen Bedingungen ausgesetzt werden (definiert sind Anzahlkonzentration, pH-Wert, Ionenstärke, DOM Konzentration und Versuchsdauer). Dispergierbarkeit kann dann definiert werden in › x% noch dispergiert nach y Stunden Beobachtungszeit. Es sind somit zwei Messungen nötig. Einfach zu beschreiben sind Proben, die bereits beim Herstellen der Vorratsdispersion innerhalb von wenigen Minuten sedimentieren und einen klaren Überstand hinterlassen. Problematisch erweisen sich hier heterogene Nanomaterialien, bei denen ein Anteil stabil dispergierbar ist, ein Anteil jedoch nicht. Im Prinzip müssten die beiden Anteile gesondert betrachtet werden.

Dispersionsstabilität – gibt an wie langzeitstabil die Dispersion unter unterschiedlichen Bedingungen ist. Wobei der Zeitraum der Beobachtung eine Rolle spielt und die Stabilität auch von der Anzahlkonzentration der ENPs in der Dispersion abhängt. Es müssen daher mindestens Beobachtungsdauer und Anzahlkonzentration definiert sein. Bei gleicher Anzahlkonzentration zu Beginn des Tests und gleicher Beobachtungsdauer werden sich unterschiedliche Materialien gleicher Dispersionsstabilität sehr ähnlich verhalten. Voraussetzung zum Vergleich unterschiedlicher Materialien ist, dass unterschiedliche Dichten berücksichtigt werden (Zentrifugationsschritt).

Die Prüfmethode benötigt eine präzise Partikelmassenkonzentrationsbestimmung im Überstand der Testgefäße. Daher wurden geeignete und häufig verwendete Verfahren (Trübungsmessung, UV/VIS-Absorptionsspektrometrie, ICP-OES und ICP-MS) auf ihre Anwendbarkeit untersucht. Die Dispersionsstabilität spielt in der technischen Chemie eine große Rolle, z.B. in der Lack- und Farbenindustrie. Hierfür existieren speziell entwickelte Messgeräte von denen der Formulaction Turbiscan LAB auf die Anwendbarkeit für das zu entwickelnde Testverfahren untersucht wurde.

Im Prinzip kann die Testung der Dispersionsstabilität als erweiterter Test der Dispergierbarkeit an mehreren Zeitpunkten angesehen werden.

Untersuchungszeitraum und Anzahlkonzentration– Der Untersuchungszeitraum wurde auf 6 Stunden festgelegt. Dieser Zeitraum ergibt sich aus den Beobachtungen, dass bei einer festgelegten Anzahlkonzentration zum Beginn des Tests die Sedimentation einer destabilisierten Dispersion nach 6 Stunden in jedem Fall abgeschlossen ist, bei Partikeln mit geringer Dichtedifferenz zum Medium wird diese durch die Zentrifugation erzielt. Die beiden Randbedingungen Anzahlkonzentration und Dauer sind daher direkt miteinander gekoppelt. Bei höherer Anzahlkonzentration wäre die benötigte Dauer geringer und umgekehrt. Hierbei ist die benötigte Genauigkeit der Anzahlkonzentration in einer Untersuchung der Empfindlichkeit der Ergebnisse von dieser Konzentration untersucht worden. Es genügt die Anzahlkonzentration im Bereich plus/minus einer halben Größenordnung zu bestimmen. Hierfür genügen meist Herstellerangaben zur mittleren Partikelgröße und Dichte um mit Hilfe einer sphärischen Geometrie die Anzahlkonzentration zu berechnen. Alternativ kann eine DLS Messung der Vorratsdispersion herangezogen werden. Aufgrund der niedrigen benötigten Genauigkeit (oder hohen Robustheit) erübrigen sich Diskussionen über die Präzision von DLS Messungen oder Herstellerangaben, die Genauigkeit der Materialdichte oder Einfluss von Partikelgeometrien. Wenn jedoch erhebliche Abweichungen von den Annahmen bekannt sind, müssen diese berücksichtigt werden. Ein Beispiel wären stäbchenförmige Partikel und Materialien mit einer sehr breiten Partikelgrößenverteilung.

Im Prinzip ist die Untersuchung der Dispersionstabilität in diesem Test eine Dispergierbarkeits-Untersuchung mit höherer Zeitauflösung. Sie zeigt zusätzlich das zeitliche Verhalten der Dispersion in Bezug auf ihre Sedimentation und Phasentrennung im natürlichen Schwerefeld, während der Zentrifugationsschritt nach 6 Stunden (Dispergierbarkeit) eine Vergleichbarkeit zwischen unterschiedlichen Materialien ermöglicht. Die höhere Zeitauflösung ermöglicht den Prozess der Trennung besser einzuschätzen (Abb. 1).

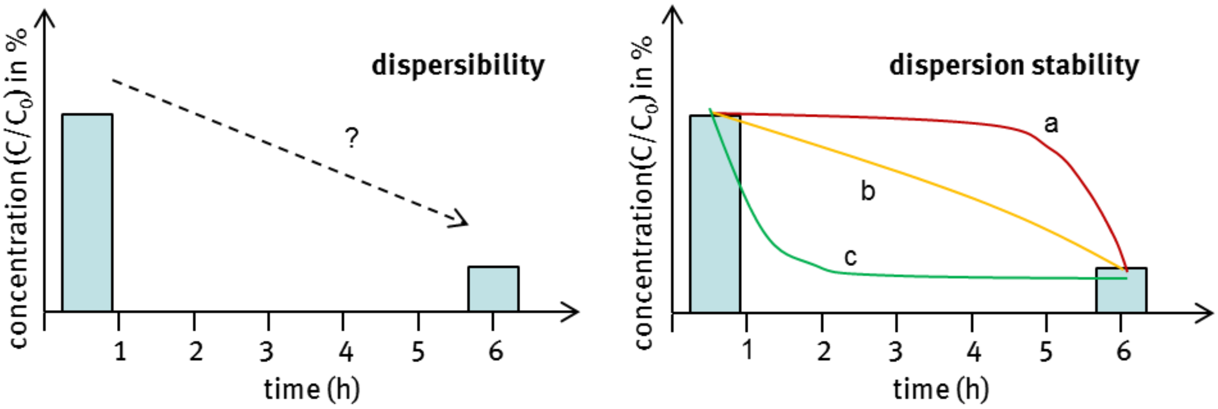


Abbildung 1: Mögliche Ergebnisse aus dem Dispergierbarkeits- und Dispersionsstabilitätstest, das 2-Punkt und 7-Punkt Messverfahren.

Der in Abb. 1 dargestellte Fall (a) würde durch Partikel entstehen, die zwar agglomerieren, aber durch ihre geringe Dichte in Schwebe bleiben, Fall (b) zeigt eine kontinuierliche Abscheidung während eines (relativ zu c) langsamen Agglomerationsprozesses. Fall (c) weist darauf hin, dass die Dispersion aus zwei Fraktionen besteht, einer stabilen, die auch über 6 h nicht agglomeriert und einer, die sehr schnell sedimentiert. Als alternative Analytikmethode wurde ein Batch-Verfahren mit einem UV/VIS Spektrophotometer erprobt, das eine noch höhere zeitliche Auflösung ermöglicht und für Partikeltypen, die im UV/VIS Spektrophotometer in Trübung oder in echter Absorption gemessen werden können, geeignet ist. Es ergeben sich Nachteile bezüglich Zeitaufwand und Empfindlichkeit, auf die in der TG eingegangen wird.

Mit Hilfe der Prüfmethode können ENM entsprechend ihres Verhaltens in verschiedene Klassen von Dispersionsstabilitäten eingeordnet werden. So wird ein ENM zunächst in Anwesenheit von DOM unter allen drei pH-Werten und Ca-Konzentrationen auf seine Dispergierbarkeit getestet (2-Punkt-Verfahren). Wenn es unter allen dieser Bedingungen zu mehr als 90% nach 6h im Überstand gefunden wird gilt es als gut dispergierbar und stabil. Wenn es zu weniger als 10% im Überstand gefunden wird, gilt es als nicht dispergierbar und instabil. Es muss dann nicht weiter hinsichtlich Dispersionsstabilität getestet werden. ENM mit Ergebnissen zwischen 10 und 90% werden dem detaillierteren Dispersionsstabilitätsverfahren unterzogen (7-Punkt-Verfahren) und unter Einfluss von DOM und ohne DOM untersucht.

Es muss darauf hingewiesen werden, dass die zum Zeitpunkt 0 h in den Testgefäßen gemessene Konzentration als 100% definiert ist und alle während des Tests auftretenden Verluste im Überstand auf diesen Wert bezogen werden. Durch Verluste an Oberflächen oder vorzeitige Sedimentation zwischen dem Ansetzen der Testdispersion und dem Start des Tests kann dieser Startwert sich vom erwarteten Wert, der sich aus Einwaage und Verdünnungsfaktoren ergibt, abweichen. Dieser Unterschied soll erfasst und im Testbericht festgehalten werden. Hohe Verluste in dieser Startphase können darauf hindeuten, dass das verwendete Gefäßmaterial für das zu testende Nanomaterial ungeeignet ist und Alternativen gefunden werden müssen.

Im Rahmen des Vorhabens wurden drei ENM ausgewählt, um die Anwendbarkeit des Testverfahrens zu überprüfen. Zum einen Silber-NPs (NM300K), die bekannt sind unter vielen Bedingungen eine stabile Dispersion auszubilden (positive Kontrolle), Kohlenstoffnanoröhren (NM400), die unter den meisten Bedingungen keine stabile Dispersion ausbilden (negative Kontrolle) und ein ENM, welche bekanntermaßen ein Dispersionsverhalten zeigt, das sehr stark von den Umgebungsbedingungen abhängt, Titandioxid (NM105).

Die ausgewählten hydrochemischen Bedingungen im Test ergeben sich aus einer Datenbankanalyse für europäische Oberflächengewässer (Salminen, et al. 2005) und aktueller Literatur zu Stabilitätsbereichen von ENM in aquatischen Medien (Hammes, et al. 2013, von der Kammer, et al. 2010, Zhu, et al. 2014). Als natürliche organische Substanz (DOM) wird Suwannee River NOM vorgeschlagen, in einer Konzentration von 10 mg/L, wobei nicht nur die Massenkonzentration der DOM und der Partikel eine Rolle spielt, sondern die verfügbare Oberfläche der Partikel in der Dispersion berücksichtigt werden soll. Der pH-Werte-Bereich wurde zunächst mit 5 – 7 – 8,5 festgelegt, im Zuge der Abstimmungen mit der OECD jedoch auf 4 bis 9 geändert, um eine Angleichung an existierende TGs zu gewährleisten. Der Bereich der Kalziumkonzentration wurde mit 0 – 1 – 10 mmol/L als $Ca(NO_3)_2$ festgelegt, mit der Option dies um $MgSO_4$ zu ergänzen (im Molverhältnis 4:1 Ca:Mg).

Die auf Basis der hier dargestellten Ergebnisse erstellte Testrichtlinie ist für die Verabschiedung durch das Council der OECD vorgesehen und kann danach auf den Internetseiten der OECD unter http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm abgerufen werden.

Summary

During the OECD *Working Party on Manufactured Nanomaterials* 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) required to perform the tests on the dispersion stability and dissolution behavior of nanomaterials in relevant environmental aquatic media. The developed TGs shall provide the experimental routine to test dispersion behavior and dissolution rate of such materials in aquatic media to estimate their possible fate in and impact on the environment and the joint GD shall answer the question of how nanomaterials should be tested under consideration of their nanomaterial specificities in further environmental tests. The current scientific report summarizes the experimental data obtained while developing the TG on dispersibility and agglomeration behavior of nanomaterials in different aquatic media as well as first considerations on the corresponding GD. Besides that a review on nanomaterials transformation reactions in environmental aquatic media is included in this report.

Engineered Nanoparticles (ENPs) may behave differently compared to solid bulk (> 1µm) materials and dissolved ions or macromolecules due to their specific surface interactions. It has been accepted that some of the existing guidelines on testing of chemicals for their environmental behavior and eco-toxicity are not applicable or require adaption for nanomaterials (OECD 2014). It is therefore required to determine if a material which has been classified as being a nanomaterial or containing nanomaterials has to be tested with specific guidelines or under consideration of specific GDs which take the differences in behavior into account. The experimental data described in this scientific report were produced in support of the development of the related TG on Dispersion Stability of Nanomaterials in Simulated Environmental Media.

Sub-micron sized particles form thermodynamically unstable dispersions in aqueous systems. However, a pseudo-stable situation might be observed when an energy barrier prevents the attachment of particles. This energy barrier, which prevents particles to agglomerate, is influenced by the particles surface chemistry and the composition of the surrounding aqueous medium. The agglomeration behavior of the particles will further determine their transport, environmental distribution, uptake by biota and final fate. While it is impossible to cover the whole complexity of environmental conditions in a laboratory test, it is possible to test the impact of major driving forces on the behavior of the particles. Currently the concentrations of manufactured nanomaterials, which will enter the environment, are expected to be far lower than the concentration of natural (nano-) particles. Therefore the assessment of environmental behavior needs to take into account the presence of natural particles and the heteroagglomeration of manufactured nanomaterials with natural particles will be a dominant pathway. However, for the decision if a material has and retains nanoparticulate properties in OECD standardized test systems the dissolution and homoagglomeration are the critical characteristics. Approaches to test ENPs for their heteroagglomeration are still poorly developed, mainly due to the fact that the counterpart for heteroagglomeration, particulate entities or surfaces of natural origin are extremely diverse and analysis of the agglomeration state of heteroagglomerates is still challenging (Hendren, et al. 2015). The approach that is presented in this report represents a simplification of extensive particle testing under various environmental conditions. The parameters that influence particle agglomeration behavior/dispersion stability are shortly described below:

Ionic strength –When particles with the similar polarity of surface charge are under investigation, high electrolyte concentration promotes their agglomeration and sedimentation.

pH –For particles with variable charge surfaces (e.g. metal oxides), with organic coatings with amphoteric character or groups which can protonate/deprotonate (e.g. carboxylic groups), the pH pays a major role for their colloidal stability. Dispersions of electrostatically stabilized particles are most unstable at the pH where this electrostatic repulsion is minimal. In the absence of any specific adsorption of

electrolytes from the dispersing medium this is the point of zero charge (PZC), when the surface charge density is zero. This is a material-related, intrinsic parameter. Often, instead of the PZC, the isoelectric point (EIP) is determined, what is the pH at which the particles do not move in an electric field. In absence of specific adsorption this IEP equals the PZC, if specific adsorption takes place the IEP is unequal the PZC and becomes an extrinsic parameter which is determined by the surrounding medium.

Concentration of Dissolved Organic Matter (DOM) –Particle dispersions generally become more stable due to adsorption of NOM on the surfaces of the particles. NOM surface adsorption may increase negative surface charge, induce charge reversal or, in cases of positively charged particles, may lead to reduction of net positive charge (up to neutralization) and therefore can also induce agglomeration. In the presence of cations strongly complexed by NOM (as Ca^{2+}) dissolved NOM may reduce the activity of the cations which will increase colloidal stability of negatively charged particles.

Particle concentration –Larger number of particles increases the number of collisions per time which leads to faster agglomeration and subsequently the sedimentation of particles.

Particle size – Large particles sediment faster. Thereby, also agglomeration directly leads to faster particle settling. Diffusion counters sedimentation, but much less for larger particles.

Particle density – Density of particles is the significant factor influencing their sedimentation rate, especially at the stage of sample's centrifugation.

Presence/absence of CO_2 in the atmosphere – Presence of CO_2 in the atmosphere above the particle dispersion can influence the stability of dispersion through changing the pH of dispersion and the properties of EDL at high pH values (above pH=5), where CO_2 is increasingly soluble in aquatic media. Adsorption of carbonate ions to the surface of the particles changes their point of zero charge.

The endpoint described in the present TG is agglomeration behavior presented as dispersion stability and dispersibility.

Dispersion stability – is the tendency of the particles in a dispersion to agglomerate and settle. It describes the behavior of the dispersion over time and how much of the particles is still dispersed after a period of time. It is in a way linked to an agglomeration rate. The agglomeration rate reports on the number of successful particle collisions per unit time, while dispersion stability is reporting the percentage of dispersed particles over time.

Dispersibility – describes how well a nanomaterial can be dispersed under defined conditions of a dispersing method. In a defined procedure the amount of dispersed particles after a given time period is measured, given a pre-set threshold (e.g. 1, 10 or 50%) the material is then termed "dispersible" or "non-dispersible".

For the test system described herein, it may be necessary to quantify nanoparticle concentrations at ppb-levels. Therefore, prior to the investigations of agglomeration behavior of nanomaterials, the detection limits of various nanoparticle detection techniques were assessed. Detection limits of ICP techniques, UV-VIS, Nephelometry and an industry-standard dispersion stability test system (Formulaction Turbiscan LAB) were tested.

Period of investigation and number concentration - The agglomeration behavior of nanomaterials can in principle be investigated by testing the sample's dispersibility. In case of such dispersibility check, only 2 samplings/ measurements have to be done. One has to be done directly after sample preparation (0 h time point) while another at the experimental endpoint (6 h time point). The time period of 6 hours was chosen empirically, as the most convenient time period to overview the particle agglomeration in the described conditions. However, the test must be performed with a certain particle number concentration in the sample. Dispersibility testing is quite convenient since it is less time-consuming, and provides a simple “Yes” or “No” answer to the question of particle agglomeration and sedimentation. However, it can cause numerous difficulties in understanding the origin of obtained data since it does not overview the dynamics of particle agglomeration/sedimentation. Such opportunity is provided by dispersion stability testing used in the presented test guideline. During dispersion stability test a number of experimental points are obtained in accordance to the particle concentration measurements performed every hour (0-6 hours). Built experimental curves can originate from similar experimental factors, but have different character. Such approach readily allows to visually overview the reasons of particle sedimentation, however it does not allow to quantitatively evaluate the data.

The choice of “dispersibility” or “dispersion stability” approaches depends on the aims of the test, and requires the understanding of advantages and disadvantages of each approach, as described above. “Dispersibility” and “dispersion stability” experimental approaches are strongly bounded to each other and are both applicable for the needs of presented TG. Thus, in certain cases, the 7-point measurements can be substituted by 2 point measurements, where measurements have to be performed at 0 and 6 hours points. Such simplified 2-point approach can be applied for the primary assessment of nanomaterial stability in aquatic media. If dispersed material reveals more than 90% (fully dispersible) or less than 10% (non-dispersible) of initial material concentration in the dispersed form after 6 hours of experimental time the 2-point measurements of material dispersibility can be considered sufficient and no further testing is required. In case the concentration of material in the dispersion after 6 hours of experimental time stays in the range of 10-90%, it is recommended to perform the full 7-point testing, it will lead to a better understanding of particle agglomeration behavior, and its reasons. Thus Figure 2 shows the possible characters of particle agglomeration (sedimentation) behavior and explains its possible reasons. Curve of (a) type would correspond to a material that sediments mainly due to the density factor. As can be observed, sedimentation mainly occurs during the last hour measurement, when particle dispersion undergoes centrifugation that facilitates sedimentation. It could mean that low density particles (as e.g. polystyrene sulfonates) agglomerate to larger clusters but still float to a large extent as they are settling slowly in the gravitational field. Centrifugation would account for this low density by applying respectively high g-forces. Given the particles have agglomerated to clusters larger the centrifugation particle size cut-off, they will sediment out in this step and an abrupt decrease in supernatant concentration is found. Curve (b) would be referred to the systems that undergo continuous agglomeration followed by particle sedimentation. This is a classic case of particle sedimentation caused by formation of agglomerates. In turn, case (c) would correspond to a dispersion containing rather heterogeneous particles. As can be seen from curve (c) the largest particles would sediment during the 1-2 hours of observations, while the smaller ones will remain the constant concentration in the dispersion. It could also mean that a fraction of 2/3 of the sample agglomerates fast and settles, while 1/3 of the particles possess higher repulsive forces and stay stably dispersed.

It has to be acknowledged that a substance may exist in several nanoforms of the same chemical identity; the different nanoforms may possess variable properties. The multi-point testing helps to reveal this heterogeneity of a substance in its different nanoforms.

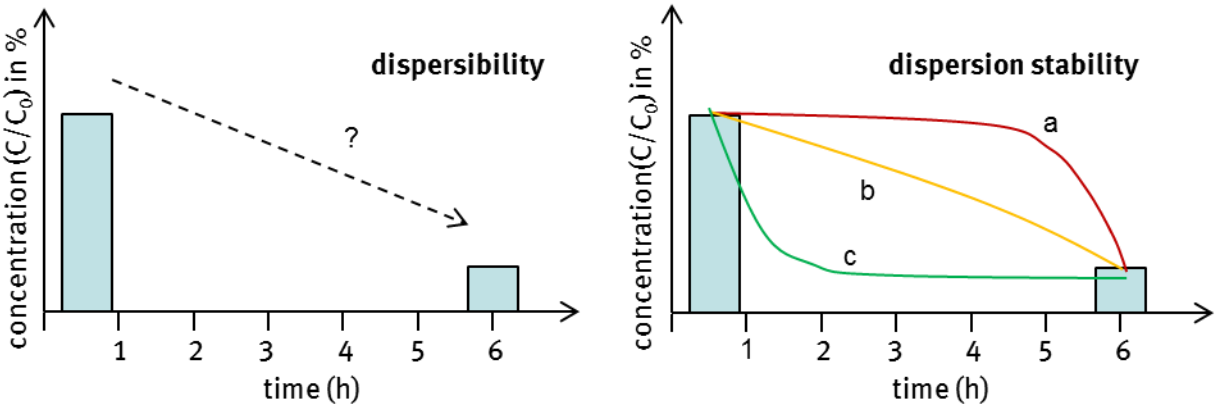


Figure 2: Possible types of particle agglomeration behavior in dispersibility test and in dispersion stability test.

Such approach, combining analysis of dispersibility and dispersion stability of investigated material can help to assess the obtained experimental data and estimate the need in further analysis with the minimal measurement cost and time spending.

Special attention has to be paid to reporting of material concentration values obtained during the measurements. The above mentioned percentage values (such as 90% or 10% of the material amount) shall be calculated in assumption that 100% of the material concentration is the amount of material measured at the initial (0 hours) measurement. This initial concentration will sometimes however be lower than the calculated (expected) material concentration, due to the material losses during experimental preparations. The difference between the calculated concentration value and the initial material concentration value (0 hours) shall be determined and reported too. The calculated concentration value shall be either derived from the amount of material (by weight) placed in the dispersion, or by full digestion of analyzed dispersion. High losses during preparation indicate that some of the instruments or lab ware used is not compatible with the dispersion. Some types of plastics do support the attachment of particles to the surfaces and promote losses.

When the direct measurements of particle concentrations during each hour is not possible due to the unavailability of suitable instruments or by some other reasons, the continuous measurements of particle concentration in the dispersion can be performed using UV-VIS spectrometry. Usage of UV-VIS spectrometry comes with certain limitations for the analysis that has to be considered during the experiments and interpretation of the results. Typical UV-VIS measurement allows a measurement of only one sample at a time. Thus the measurement of each sample will demand a continuous work of UV-VIS spectrometer for 24 hours. No replicate measurements would be possible during such measurement. However, the number of measurements over time is dramatically increased (a one measurement per 30 seconds would be a typical acquisition rate) and can be easily regulated to the desired value. This even increases accuracy due to a large number of replicates which differ only very little in their acquisition time point. Here also an improved time-resolution is obtained, which might sheed even more light on the agglomeration process than the 7-point testing. Particle concentration can be monitored by UV-VIS spectrometer based on light scattering or light absorption by the particles, depending on the properties of the particles. Here NM300K and NM105 have been monitored in absorption because both particles have true absorption bands, the silver particles from the plasmon resonance and the TiO_2 particles from the UV-filter properties of the material. Results and sensitivities reported here will differ significantly from materials like SiO_2 nanoparticles where only weak scattering will produce a (small) signal in the photometer. The signal to mass ratio for light scattering is typically much lower than for true absorption. Detection wavelengths have to be chosen with care and a spectral scan would be advised before selecting the wavelength. The final results are presented in the form of relative to the first measurement absorption, (A/A0).

The development of the experimental procedures was based on three different particle types. They each represent a positive and negative control as well as a borderline case. Ag-NPs (NM300K, OECD material) has been used as positive control that shows good dispersion stability under almost all environmental conditions considered in the test, NM400 carbon nanotubes have been chosen as a negative control with vanishing dispersion stability under almost all conditions and NM105 TiO2-NPs have been employed as a borderline case which shows good and no stability, depending on the water chemistry.

The parameters in the aquatic media in which the stability is tested have been selected according to database analysis (Salminen, et al. 2005) and current literature on NP stability in aqueous media (Hammes, et al. 2013, von der Kammer, et al. 2010, Zhu, et al. 2014).It has become evident that if comparing the 90% percentile of surface water concentrations of stability controlling ions with their de-stabilizing potential, the major role is played by divalent cations, NOM and pH. These three parameters have been introduced into the experimental procedure in different concentrations or values. NOM is only applied in two concentrations (0 and 10 mg/L, depending on the surface area of the particles).

The OECD TG developed based on the research presented in the report is intended for OECD council adoption and will then be available at the webpage on OECD Guidelines for the Testing of Chemicals: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

1 Introduction

The steadily increasing production and spreading usage of manufactured nanomaterials have always been seen to increase the probability of finding them in natural systems, especially aquatic ones. This fact has risen a discussion, weather the existing regulatory protocols and related risk assessment are sufficient and adequate to determine the fate of manufactured nanomaterials and their impact on the natural environment.

Agglomeration behavior was identified as an important parameter affecting the environmental behavior of nanomaterials. This parameter depends on physicochemical characteristics of the dispersion media, dispersion preparation, (number-) concentration and polydispersity of the nanomaterial and concentration of other substances and particles in the dispersion, physicochemical characteristics of the nanomaterial itself and type of interaction between nanoparticles. Among all forces existing between particles, there are four major forces of different nature acting within the dispersion, controlling the particle interactions. These are the forces of electrostatic interactions (attractive or repulsive), forces of Van der Waals attraction, forces of steric stabilization from surface adsorbed macromolecules and magnetic forces in-between the particles revealing magnetic properties. Due to the forces acting in-between the colloidal particles they can form (meta-) stable dispersions or undergo the processes of agglomeration. Due to always attractive Van der Waals forces, lyophobic dispersion colloids, in which category most of the known nanomaterials fall, agglomerate as long as no stabilizing, repulsing forces between particles are present. A stable dispersion therefore always represents a non-equilibrium situation which is kinetically hindered to reach equilibrium in the agglomerated state. Formed agglomerates (working definition from ISO TS27687 2008) (Standardization 2008) are described as collection of weakly bound particles where the resulting surface area is similar to the sum of surface areas of initial individual particles. Thus, structures formed by attachment of particles to each other from a formerly dispersed stage would likely be termed agglomerates. Thus, information on nanomaterials agglomeration behavior in aquatic media is, beside e.g. dissolution rate, one prerequisite for a proper further environmental testing of nanomaterials and interpretation of test results.

During the OECD meeting in Berlin in January 2013 (OECD 2014) experts agreed on the necessity to develop two new OECD Test Guidelines and a corresponding guidance document required to perform the tests on the agglomeration/dispersion and dissolution behavior of nanomaterials in relevant environmental aquatic media. The developed TGs shall provide the experimental routine to test agglomeration/dispersion behavior and dissolution rate of such materials in aquatic media to estimate their possible fate in and impact on the environment and answer the question of how nanomaterials should be tested under consideration of their nanomaterial specificities in further environmental tests. The current scientific report summarizes the experimental data obtained while developing the TG on agglomeration behavior (i.e. dispersibility and dispersion stability) of nanomaterials in different aquatic media as well as first considerations of the corresponding GD. Besides that a review on nanomaterial`s transformation reactions possible in environmental aquatic media is included in this report.

Since the aim of this study is to develop an approach that would allow determining the dispersion behavior in the environmental aquatic media, the choice of the media for the preparation of dispersion is the task of paramount importance. The system shall of course represent natural surface waters and at the same time be possibly simple to be conducted in a reasonable amount of time in the laboratory. A concept that allows doing so was proposed by F. von der Kammer (von der Kammer, et al. 2010) and further developed and checked for environmental relevance by Ottofuelling et al. (Ottofuelling, et al. 2011). It is based on the construction of a multi-dimensional test matrix including environmentally relevant test parameters predominantly influencing nanoparticle agglomeration. These parameters include presence of various cations and anions, organic matter, pH, concentration of particles, their size, density, and surface chemistry, presence or absence of CO_2 in the surrounding atmosphere, etc.

Such an approach is also utilized in the present study, but broken down to the parameters and parameter ranges identified as relevant (covering more than 90% of the values observed in ~800 surface water samples across Europe, extracted from the FOREGS Geochemical Atlas of Europe). This experimental routine should cover the important hydrochemical situations while being less complex. The parameters that influence particle agglomeration behavior and dispersion stability are described below:

Ionic strength – Presence of electrolyte in the aquatic media is essential for the formation of the Electric Double Layer (EDL), the ionic structure in the medium appearing close to material surface as response to the surface charge of particles. When particles with the opposite surface charge are considered, low electrolyte concentration leads to faster particles agglomeration and sedimentation. In this case increased thickness of EDL enables particles to approach each other and interact faster through forces of electrostatic attraction. Oppositely, when particles with the similar sign of surface charge are under investigation, high electrolyte concentration promotes their agglomeration and sedimentation. Compact, thin EDL (at high electrolyte concentration) allows particles to approach closer to each other and interact more effectively, minimizing forces of electrostatic repulsion. Counter-ions (those countering the potential on the surface of the particles) with higher valencies cause stronger de-stabilization of the dispersion as would be expected from the ionic strength alone (Schultze-Hardy Rule).

pH – pH has a crucial effect on particle agglomeration by influencing the surface charge of particles which are carrying variable charges at the surface. Dispersions of particles are most unstable at the pH of Point of Zero Charge (PZC). At this pH the net charge at the particle surface that forms EDL is minimal. As a result the inter-surface electrostatic repulsion forces are minimal, that allows particles to aggregate and sediment. Variable charge surfaces show positive potential at pH below and negative potential at pH above the PZC.

Concentration of Dissolved Organic Matter (DOM) – DOM influences the stability of particle dispersions through adsorption on the particle surfaces. Particle dispersions generally become more stable due to such interactions. However, when the adsorbed DOM just balances a positive charge on the particles surfaces, agglomeration could be observed. DOM molecules in the liquid phase also act as a buffering agent, keeping the pH of particle dispersion at a constant level. In the presence of cations strongly complexed by NOM (as Ca^{2+}) dissolved NOM may reduce the activity of the cations which will increase colloidal stability of negatively charged particles.

Particle (number) concentration – For dispersions of particles, particle concentration is typically given in mass concentration of particles per volume of dispersion liquid. However especially the number concentration of particles plays an important role for the number of particle collisions in dispersion per unit time, therefore for the speed of the agglomeration process and for the dispersion stability. Large numbers of particles in the dispersion increase the probability of inter-particle collisions. Larger number of collisions per time leads to faster agglomeration and subsequently the sedimentation of particles. Particle number concentrations are related to mass concentrations via particle density and size (and geometry of the particles).

Particle nature and surface chemistry – The surfaces of various particles are terminated with surface functional groups which determine the surface chemistry of the particles in the dispersion. The amount and type of surface groups determine the extent in which particles are able to develop surface charge and build EDL. These two features play crucial role in particle dispersion stability as described above. Surface-bound molecules may introduce a non-electrostatic stability factor, the steric stabilization, where the molecules prevent attachment of the particles by creating a steric repulsive barrier. If sterically stabilizing molecules are only weakly bound to the surface, dilution of the particles in dispersion can lead to desorption of the molecules, which can further lead to de-stabilization of the dispersion through particle agglomeration.

Particle size – Large particles sediment faster. Thereby, also agglomeration directly leads to faster particle settling. Diffusion counters sedimentation, but much less for larger particles.

Particle density – Density of particles is the significant factor influencing their sedimentation rate, especially at the stage of sample's centrifugation. Because of this, present guidelines are applicable for the particles with the density not smaller than 1 kg/m^3. Particles with high densities (as e.g. Gold-NPs) have relevant settling velocities in water already below 1 µm particle size. They do not necessarily need to agglomerate to produce gradually decreasing concentrations in the supernatant over the experimental duration. However, they also do not form stable dispersions in water.

Presence/absence of CO_2 in the atmosphere – Presence of CO_2 in the atmosphere above the particle dispersion can influence the stability of dispersion through changing the pH of dispersion and the properties of EDL at high pH values (above pH=5), where CO_2 is increasingly soluble in aquatic media. Dissolved carbonate ions likely take part in adsorption at the mineral surface in a form of counter ion, changing the surface charge, the point of net zero proton charge (not to be confused with the isoelectric point measured by zeta potential measurements) and thereby the properties of EDL, consequently influencing the dispersion stability.

The parameters in the aquatic media in which the stability is tested have been selected according to database analysis (Salminen, et al. 2005) and current literature on NP stability in aqueous media (Hammes, et al. 2013). It has become evident that if comparing the 90% percentile of surface water concentrations of stability controlling ions with their de-stabilizing potential, the major role is played by divalent cations, NOM and pH. These three parameters have been introduced into the experimental procedure in different concentrations or values. NOM is only applied in two concentrations (0 and 10 mg/L, depending on the surface area of the particles).

2 Development of the Test Guideline, dispersion stability testing.

The general layout of the dispersibility and dispersion stability tests was defined prior to the experimental phase. Both, dispersibility and dispersion stability refer in principle to the behavior of dispersions and therefore a homoagglomeration test is in focus where particles react with each other and not with entities of another kind. Although the environmental behavior is more thought to be determined by heteroagglomeration with other natural particles, due to the low expected concentrations of manufactured nanoparticles in the environment, a material characteristic might be better determined from an operationally defined homoagglomeration test since the conditions are easier standardized.

In principle, the stability of a dispersion can be directly determined by analyzing the agglomeration rate constant in e.g. a DLS instrument. However, the fact that only one condition per time can be analyzed in one instrument lead to the conclusion that a multi-parameter vial testing, as it had been successfully demonstrated in (Ottofuelling, et al. 2011) and recently been demonstrated even for heteroagglomeration in (Barton, et al. 2014) is more suitable. The experimental procedure is based on the following steps:

(1) dispersion of powdered material in ultrapure water (MQ) or preparation of a stock from a liquid dispersion to obtain a stock dispersion with known mass concentration

(2) subjection of the nanoparticles to different hydrochemical conditions at defined (particle number) concentration in a reaction vessel (test dispersion, centrifugation vial) for a defined time period (here: 6h)

(3) measuring the remaining concentration in the topmost part of the test dispersion at defined time points (dispersion stability) or at a final time point (dispersibility)

(4) the last measurement point (here at 6h) is performed with a centrifugation step that is calculated according to particle density and desired size cut-off (e.g. 1µm) to fully remove agglomerated particles which have not fully settled out of the region where the sample for analysis is taken and independent of their density.

Figure 3 shows the difference between a dispersibility test and a dispersion stability test. Dispersibility and dispersion stability experiments follow the same procedure, with the dispersion stability test providing more data points over the progressing reaction of agglomeration and settling. This allows more insight into the character of the process. If this information is not needed, the 7-point measurements can be substituted by 2-point measurements, where measurements have to be performed only at 0 and 6 hours. Such simplified 2-point approach can be applied for the primary assessment of nanomaterial dispersibility in aquatic media of various composition.

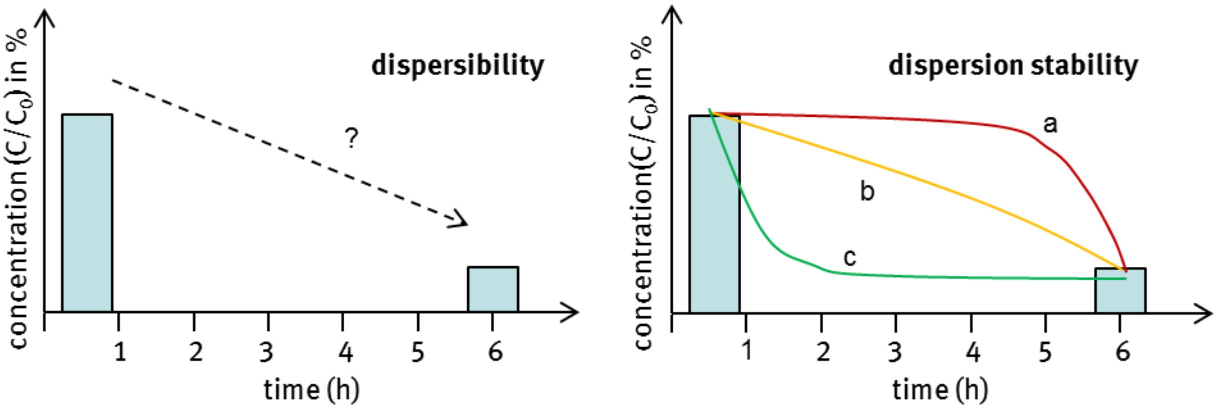


Figure 3: Possible types of particle agglomeration behavior in dispersibility test and in dispersion stability test.

If dispersed material reveals more than 90% (equals “fully dispersible”) or less than 10% (equals “non-dispersible”) of the initial material concentration in the dispersed form after 6 hours of experimental time the 2-point measurements of material dispersibility can be considered sufficient. In case the concentration of material in the dispersion after 6 hours of experimental time stays in the range of 10-90%, it is recommended to perform the full 7-point testing, it will lead to a better understanding of particle agglomeration behavior, and its reasons. Figure 3 (dispersion stability) shows three possible characters of particle agglomeration (sedimentation) behavior. Curve of (a) type would correspond to a material that sediments mainly due to the density factor. As can be observed, sedimentation mainly occurs during the last hour measurement, when particle dispersion undergoes centrifugation that facilitates sedimentation. It could mean that low density particles (as e.g. polystyrene sulfonates) agglomerate to larger clusters but still float to a large extent as they are settling slowly in the gravitational field. Centrifugation would account for this low density by applying respectively high g-forces. Given the particles have agglomerated to clusters larger than the centrifugation particle size cut-off, they will sediment out in this step and an abrupt decrease in supernatant concentration is found. Curve (b) would be referred to the systems that undergo continuous agglomeration followed by particle sedimentation (particles have a density ›› than 1). This is a classic case of particle sedimentation caused by formation of agglomerates. In turn, case (c) would correspond to a dispersion containing rather heterogeneous particles. As can be seen from curve (c) the largest particles would sediment during the 1-2 hours of observations, while the smaller ones will remain at a constant concentration in the dispersion. It could also represent a situation where a fraction of 2/3 of the sample agglomerates fast and settles, while 1/3 of the particles possess higher repulsive forces and stay stably dispersed.

It has to be acknowledged that a substance may exist in several nanoforms of the same chemical identity; the different nanoforms may possess variable properties. The multi-point testing helps to reveal this heterogeneity of a substance in its different nanoforms. To determine the best experimental procedure several points were considered:

- **standardization of the energy input across the different dispersion instruments**
    - bath or probe sonication
    - by the nominal power output of the device
    - by instrument readouts
    - by calorimetric measurements in simple settings
- **influence of the dispersion procedure on the outcome of the test**
    - influence of the start concentration on the optimal duration of the test (particle number concentrations)
    - influence of pre-wetting of samples
    - influence of atmospheric versus inert atmosphere experiments (influence of CO_2)
- **are the pH, electrolyte and NOM type and concentrations reasonable?**
    - pH range and pH stability – feasibility of the approach
    - buffered versus unbuffered systems (pH shift due to CO_2 intrusion)
    - ionic strength range
    - NOM type and concentration range
    - suitability of different types of NOM (purity, agglomeration under high ionic strength and low pH)
- **how can the stability be assessed by analyzing the supernatant?**
    - required detection limits
    - suitability of techniques for different types of particles
    - comparison of different techniques
- **can stand-alone, industry standard instruments like Formulaction Turbiscan be used for the purpose of stability testing under the chosen conditions?**

For proof of principle three types of nanomaterials (NM400, NM105 and NM300K) were investigated under various conditions and procedures and the effect on the test outcome was assessed. The materials represented a fully dispersible material (silver NPs, positive control, NM300K) and a borderline case (TiO_2-NPs, NM105). The negative control (carbon nanotubes, NM400) was also tested under different conditions and appeared, as expected, as not dispersible.

2.1 Materials and methods

2.1.1 Tested materials

Three nanomaterials which might be used as possible reference substances were used to underpin the applicability of the proposed experimental routines as being positive and negative controls plus a borderline case. These nanomaterials were Ag (NM300K) (Klein, et al. 2011) nanoparticles, Carbon nanotubes CNTs (NM400) (Rasmussen, et al. 2014a) and TiO_2 (NM105) (Rasmussen 2014b). All materials were provided by JRC nanomaterials repository (European Commission). Dispersions of Ag (NM300K) nanoparticles were completely stable over time under almost all test conditions, while dispersions of CNTs were not stable through entire test conditions. Such dispersion properties were anticipated from available data at the start of the project and the results of the applied test routines confirmed these assumptions (data not shown). It is recommended using those as positive and negative controls in the proposed TG.

Dispersions of TiO_2 (NM105) nanoparticles showed variable stability depending on test conditions (data not shown), extensive tests on NM105 under various hydrochemical conditions are reported in Ottofuelling et al.(Ottofuelling, et al. 2011). Therefore this material was accepted as an appropriate nanomaterial for the development of the Test Guideline and for quality control in further testing of nanomaterials for inter-laboratory comparison.

In addition, Ag (NM300K) was included in the experiments as control representing a stable dispersed nanomaterial.

2.1.2 Materials, chemicals and used equipment

Used laboratory equipment, included but was not limited to:

(a) Pipets for sample preparation (5ml, 2ml, 0.1ml volume), Eppendorf

(b) Ultrasonic probe for homogenization of particle dispersion, Bandelin Sonopuls 497 Titanteller TT13

(c) pH-meter to measure the pH of the dispersion

(d) DLS device for measuring the particle size (Malvern ZetaSizer ZS), 1 cm polycarbonate cuvettes

(e) 50 ml conical bottom polypropylene centrifuge tubes (N) to perform the agglomeration experiments

(f) 10 ml polypropylene tubes (N) to prepare the samples for ICP-OES/MS analysis

(g) Standard lab centrifuge, swing-out rotor, capable of 3500g relative centrifugal force (rcf)

(h) Inductively Coupled Plasma Mass Spectrometry Device for analysis of AgNP nanoparticle dispersions (ICP-MS), Agilent Technologies 7700, 8800 and 7900 ICP-MS

(i) Inductively Coupled Plasma Optical Emission Spectrometry Device for analysis of TiO_2 nano particle dispersions, (ICP-OES) Perkin Elmer Optical Emission Spectrometer Optima 5300 DV

(j) Ultraviolet-visible spectroscopy (UV-VIS) spectrometer, Perkin Elmer Lambda 35

(k) Nephelometer (nephelometry/turbidity measurements), 2100AN IS Turbidimeter

(l) Turbiscan (vertically resolved turbidity measurements), Formulaction Turbiscan lab

Materials:

(a) Water (H_2O) – ultrapure DI (18 Ohms resistivity) (MQ)

(b) Sodium Hydroxide solution (NaOH) – 0.1M solution in ultrapure DI water, for pH establishing

(c) Hydrochloric acid (HCl) – 0.1M solution in ultrapure DI water, for pH establishing

(d) NOM solution- solution of Natural Organic Matter (1g/L), SRNOM (Green, et al. 2014)

(e) Calcium Nitrate ($Ca(NO_3)_2$) – 0.1M solution to establish the needed concentration of electro lyte

(f) TiO_2 (NM105) nanomaterial

(g) Ag (NM300K) nanomaterial

(h) CNT (NM400) nanomaterial

2.1.3 Application of instrumental techniques

One of the major considerations in a dispersibility test would be to define the threshold value for the concentration of stable dispersed particles after the experimental period that would assign the material as dispersible or not. One reasonable threshold could be 1% of the initial concentration. To meet the standardized number concentration in the beginning of the test, very small particles might then be applied at a starting mass concentration of as low as 1 mg/L. This requires analytical techniques that are able to quantify NP dispersions down to 10 µg/L (1% of starting concentration) and below. To be able to choose the most reliable, sensitive and convenient method to investigate the nanomaterial agglomeration behavior, several analytical techniques were tested. At first, analytical techniques such as ICP-OES/MS, UVVIS spectrometry, Turbiscan and nephelometric turbidity were tested for their limits of nanoparticle detection. For these purposes sets of samples with various nanoparticles concentrations were prepared and analyzed until the smallest concentration that could not be properly detected by the device. At second, techniques that revealed satisfactory detection limits were applied for time resolved tracking of nanomaterial agglomeration behavior. These were ICP-OES/MS and UVVIS techniques. Finally, ICP-OES/MS techniques were chosen as the most sensitive and convenient, allowing the investigations of inorganic nanoparticle agglomeration behavior within convenient time-frames and sample-loads.

2.1.4 General experimental conditions

All described experimental conditions were chosen to serve two main purposes:

(a) They must resemble types of electrolytes and conditions or concentration ranges of natural waters which are controlling dispersion stability and were easy to reproduce in the laboratory environment. All experiments were performed in triplicates at conditions close to standard (298.15 K (25 °C, 77 °F), 101.325 kPa (14.7 psi, 1.00 atm, and 1.01325 bar)).

(b) The specific concentrations of stocks and samples were chosen to ensure easy handling during experimental procedures and instrumental analysis. For preparation of dispersions 50 ml plastic tubes (polypropylene, 50 mL conical bottom centrifuge tubes, 3 cm in diameter, 11.5 cm tall) were used.

The experimental conditions were chosen to represent the major drivers that control stability of dispersions in natural water in a most simple way while covering most of the encountered conditions in the aquatic environment.

The pH is a major driver for controlling the pH-variable charge on the surface of NPs. In European stream waters pH values between 5 and 8.5 cover 97% of the pH values encountered (FOREGS Geochemical Atlas of Europe). Multivalent cations lead to dispersion destabilization of negatively charged NPs at by far lower concentrations than mono-valent cations, what is known as the Schultze-Hardy Rule. When the critical coagulation concentrations (CCC) for monovalent cations range at 100 mmol/L, the CCC of divalent cations is around 2 mmol/L and the CCC of trivalent cations is even lower. Looking at the actual concentration ranges of monovalent, divalent and trivalent cations in surface waters, it becomes evident that divalent cations are the most important ones and can simply be represented by Ca^{2+} salts. The median concentrations of major stream water components in the Europe are: Na^+: 0.29 mmol/L, K^+: 0.04 mmol/L, Ca^{2+}: 1 mmol/L, Mg^{2+}: 0.25 mmol/L, NO_3^-: 0.05 mmol/L, SO_4^{2-}: 0.16 mmol/L, HCO_3^-: 0.47 mmol/L. Here we have chosen calcium nitrate as the major electrolyte, however it could be shown in Ottofuelling et al. (2011) that sulfate has a strong agglomeration effect on TiO_2 NPs for positively and negatively charged particles. Waters containing more than 0.1 mmol/L sulfate will be better represented in the test when calcium sulfate is used as electrolyte. It must be noted that 90% of European stream waters are below 1 mmol/L of sulfate, while 50% are below 1 mmol/L with regard to calcium. With calcium sulfate a strongly agglomerating divalent anion would be over-represented compared to calcium in the test systems. To avoid this we (as stated above) decided for a nitrate as anion.

Although the nitrate concentration in the 1 mmol/L electrolyte test is close to the maximum of all nitrate levels in stream waters, the effect on agglomeration is negligible at this level.

In test vials which are open to the atmosphere and only contain the electrolyte and the NPs, the buffer capacity against adsorbed CO_2 (lowering of the pH) is negligible. Therefore setting of a precise pH might become difficult and the pH values will not be stable over time. To counter this, a pH-buffer might be used. However, this will add electrolytes to the test system, which are not environmentally relevant and add to the ionic strength. Therefore it was decided to allow two buffer systems to achieve stable pH conditions: $NaHCO_3$ and addition of DOC in the form of a purified NOM or humic acid. The addition of 5 mmol/L $NaHCO_3$ is about the 90% percentile of environmental concentrations but a strong effect on stability is not suspected, because of the monovalent character. Nevertheless carbonate ions may adsorb to the NPs surface and increase the portion of negative charge (either reducing a net positive or increasing net negative charge).

When adding NOM to the test systems the organic matter will interact in various ways with the NPs and the test solution. Adsorption to NPs surface will increase the portion of negative charge (either reducing a net positive or increasing net negative charge, depending on the NOM to surface area ratio and the original surface chemistry, e.g. charge density). This will change the electrostatic interaction and induce in most cases an increase in dispersion stability. The adsorbed molecules will also add a steric stabilization effect. In the presence of Ca^{2+} ions the NOM will complex the calcium ions thereby reducing the concentration of the free ions, hence their activity and effect on agglomeration.

It must be taken into account that all NOM brings impurities into the test system. Depending on the quality of the NOM, the source material and the efforts taken to purify the NOM, different amounts of metals and anions are introduced into the test solution (see table 5 and 6). These add to the already present electrolyte and may influence dispersion stability, especially at elevated DOM concentrations.

2.1.5 Preparation of nanoparticle stock dispersions (TiO_2, Ag, CNTs)

0.1 g of TiO_2 (NM105) material was pre-wetted in 1 ml deionized water and left for 24 h to ensure the proper interaction of material surface with water. After 24 h of pre-wetting the resulted wet paste was dispersed into 100 ml of MQ water, thus providing a total concentration of 1 g/L (1000 ppm). Resulting dispersion was sonicated for 10 minutes at the power level of 40 W to ensure proper dispersion and homogenization.

The stock solution of Ag (NM300K) nanoparticles with the initial concentration of ~10% was diluted down to the concentration of 100 ppm. Resulting dispersion did not require any additional preparations (sonication, etc.) and was used directly as a stock dispersion for the further sample preparation.

CNTs (NM400) were bath and tip sonicated for various times, at 1000 and 50 mg/L in MQ and with different concentrations of NOM (10 and 30 mg/L). In no case a stable stock dispersion could be achieved. The dispersion separated quickly (between 10 and 120 min until a clear supernatant was visible above a sediment and even directly after sonication large clumps of the material were floating in the dispersion media. We categorized the material as non-dispersible under all conditions.

2.1.6 Preparation of DOM stock solution

DOM influences the stability of particle dispersions through adsorption on the particle surfaces, complexation of cations and providing steric stabilization effect. In general particle dispersions become more stable due to such interactions. However, when the adsorbed DOM balances the positive charge on the particles surfaces, agglomeration could be observed. Thereby the positively charged particle surfaces are not to be expected in tests that contain DOM. DOM molecules in the liquid phase also act as a buffering agent, keeping the pH of particle dispersion at a constant level. For the purposes of the presented test guidelines Suwanee River Natural Organic Matter (IHSS Suwannee River NOM, SRNOM)) was chosen as the commercially available and strictly standardized material with the known content of Dissolved Organic Carbon (DOC).

Stock solution of 2R101N Suwannee River NOM (SRNOM) (1000 ppm NOM) was prepared and characterized for DOC content. 250 mg of SRNOM was weighted and diluted in the 250 ml of MQ water, the resulted solution was kept in brown glass closed vessels. After adjustment of pH to pH=8.5-9, NOM solution was left under vigorous stirring for 24 hours. After that the pH was measured and adjusted to the same values (pH=8.5-9) if it was needed. The solution was filtrated using a vacuum set-up, consisting of pump and bottle-top 0.2 µm PES filter. Thereafter the solution of NOM was analyzed for the DOC content. When the described procedure was followed, the DOC content normally remained within the range of 400-500 ppm. Resulted solution was kept in the fridge at 4°C in brown-glass bottles avoiding exposure to the light.

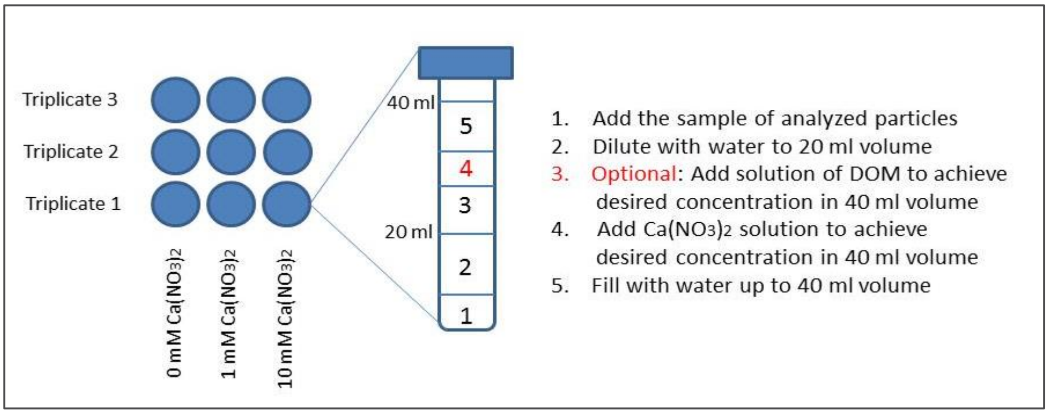
2.1.7 Experimental design applied for testing of nanomaterials dispersion behavior in presence of electrolyte and NOM (in presence or absence of CO_2) by ICP-OES/MS techniques.

To study the agglomeration behavior of particles in dispersion in presence of only electrolyte (no DOM, further on denoted MQ media), the aliquots of sonicated TiO_2 (NM105) stock dispersion (2 ml) or stock Ag (NM300K) nanoparticle dispersion (0.4 ml) were taken into 50 ml plastic tubes and diluted with deionized water to the volume of 20 ml. Dilution of dispersion of tested material was made to minimize the contact of particles in dispersion with concentrated solution of electrolyte, added in the next step. Stock solution of electrolyte with concentration of 100 mM was then added to the previously diluted dispersion of tested material (20 ml), optionally containing solution of DOM, in amounts of 0, 0.4, and 4 ml to achieve the final electrolyte concentrations of 0, 1, and 10 mM respectively. The samples were filled with MQ water up to 40 mL.

To test the dispersion stability of the particles in presence of DOM (further on denoted DOM media), the aliquots of sonicated TiO_2 (NM105) stock dispersion (2 ml) or stock Ag (NM300K) nanoparticle dispersion (0.4 ml) were taken into 50 ml plastic tubes and diluted with deionized water to the volume of 20 ml. Necessary amount of SRNOM stock solution was added to the samples to achieve the desired concentration of DOC = (30 ppm) in the final volume of the samples (40 ml). Stock solution of electrolyte with concentration of 100 mM was then added to the previously diluted dispersion of tested material (20 ml) in amounts of 0, 0.4, and 4 ml to achieve the final electrolyte concentrations of 0, 1, and 10 mM respectively. The samples were filled with MQ water up to 40 mL.

The concentration of DOC=30 ppm was chosen because it covers the 95% percentile of DOC concentrations found in surface waters (for data see the FOREGS Geochemical Atlas database (Salminen, et al. 2005)). In case of TiO_2, NM105 nanomaterial, DOC reveals the dispersion stabilizing properties down to the concentration of 1.5 ppm. To maintain comparable situation in tests the amount of DOC in the samples of interest should be based on the surface area of the analysed particles in the sample. For each 1 m^2 of material surface in the dispersion, 0.004 g of DOC is recommended. This value originates from the assumption that at least 1 molecule of DOM per 1 nm^2 of particle surface area is needed to provide the successful stabilization of dispersion. 1 m^2 can be converted to 1×10^{18} nm^2 that results into same number (1×10^{18}) of DOM molecules needed for the stabilization. Assuming the Molecular mass of NOM to be approximately 2000 g/mole and knowing Avogadro's number as 6.02×10^{23} one can calculate the necessary amount of DOM (g) per 1 m^2 that equals to 3.3 mg. This value in turn can be converted into 1.65 mg of DOC per 1m^2, (3.3 divided by 2) since DOM contains about 50% of carbon by weight. A certain safety margin being required, the obtained value is increased approximately 2.5 times, that provided the result of 0.004 g of DOC per 1 nm^2.

The influence of CO_2 on the dispersion stability was checked both for electrolyte- and electrolyte/DOM-containing systems. To check whether the dispersions of analyzed materials were stable in presence or absence of CO_2 all solutions used in experimental procedures were degassed. Thus the dispersion of nanomaterials, solutions of electrolyte and NOM underwent degassing that was performed by vigorous bubbling N_2 gas through the solutions for at least 2 hours. Prior and during the experimental procedures all solutions and samples were kept in closed vessels and tubes sealed with Parafilm when not used. Possible experimental design, common for experiments described above, is presented in figure 4.



1. Add the sample of analyzed particles
2. Dilute with water to 20 ml volume
3. Optional: Add solution of DOM to achieve desired concentration in 40 ml volume
4. Add $Ca(NO_3)_2$ solution to achieve desired concentration in 40 ml volume
5. Fill with water up to 40 ml volume

Figure 4: Possible experimental design to investigate electrolyte and DOM influence on dispersion behavior of nanoparticles in presence and absence of CO_2.

To study the dispersion behavior of tested nanomaterials in the media with the establish pH, the general experimental approach (excluding addition of DOM solution) was adopted. Prior to bringing the volume of the samples to 40 ml, $NaHCO_3$ solution was added to the sample to achieve the concentration of 5 mM in the samples. $NaHCO_3$ was used as a buffering agent to provide the stability of established pH values. After bringing the volume of samples to 40 ml, the required pH of samples media was established by addition of negligible amounts of concentrated HCl or NaOH. The possible experimental design is presented in figure 5.

1. Add the sample of analyzed particles
2. Dilute with water to 20 ml volume
3. Add $Ca(NO_3)_2$ solution to achieve desired concentration in 40 ml volume
4. Add $NaHCO_3$ solution to achieve 5 mM concentration in 40 ml volume
5. Fill with water to 40 ml volume

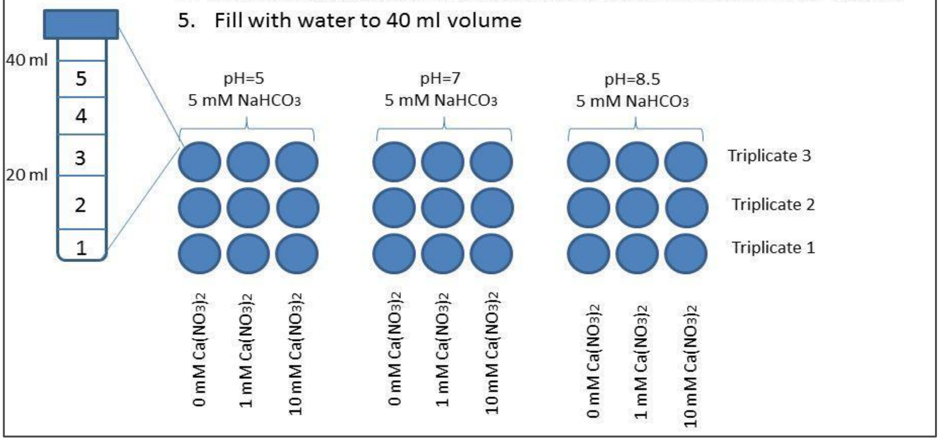


Figure 5: Possible experimental design to investigate nanoparticle dispersion behavior at fixed pH.

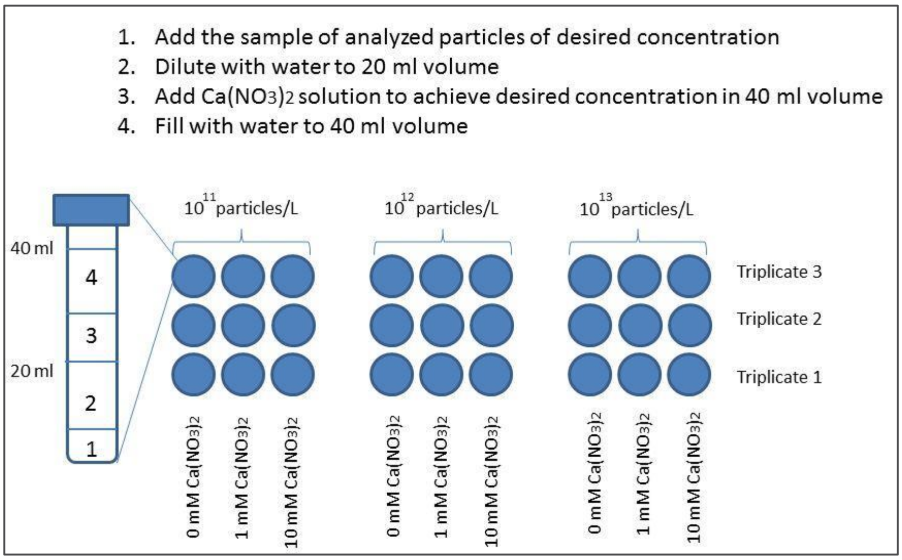
The influence of CO_2 on the dispersion stability was checked both for electrolyte- and electrolyte/DOM-containing systems. To check whether the dispersions of analyzed materials were stable in presence or absence of CO_2 all solutions used in experimental procedures were degassed. Thus the dispersion of nanomaterials, solutions of electrolyte and NOM underwent degassing that was performed by vigorous bubbling N_2 gas through the solutions for at least 2 hours. Prior and during the experimental procedures all solutions and samples were kept in closed vessels and tubes sealed with Parafilm when not used. However for the tested materials no influence of the presence or absence of CO_2 could be observed.

2.1.8 Experimental design applied for testing nanomaterial dispersion behavior in dispersions with different particle concentrations

To study the effect of different particle number concentrations at the start of the test, dispersion behavior of tested nanomaterials in the media with established particle number concentration, general experimental approach described in section 5.7 (excluding addition of DOM solution) was adopted. The particle number concentration was calculated according to the formula:

$$\mathrm{N} = \frac{\mathrm{M}}{P \times \mathrm{V}}$$

Where N is the number of particles in the dispersion, M is the mass of the powder measured to prepare the dispersion, P (kg/m³) is the density of the particle material (known value), V (m³) is the volume of particles calculated either from DLS size-measurements (we applied the formula for the volume of a sphere and the first mode of the intensity weighted distribution, not the Z-average) or by other means (microscopy) (would require specific formulas for volume calculations). Possible experimental design is presented in figure 6.



1. Add the sample of analyzed particles of desired concentration
2. Dilute with water to 20 ml volume
3. Add $Ca(NO_3)_2$ solution to achieve desired concentration in 40 ml volume
4. Fill with water to 40 ml volume

Figure 6: Possible experimental design to investigate nanoparticle dispersion behavior in dispersions with various particle concentrations.

2.1.9 Time resolved sample analysis by ICP-OES/MS

To check the dispersion stability in the prepared samples (40 ml), the remaining particle concentration in the top 0.5 cm volume of dispersion was determined. For a fully stable dispersion, this value shall not change over time, while agglomeration and as a consequence settling shall gradually reduce the concentration of particles in the top 0.5 cm volume of the sample. The aliquots of the supernatant of the samples were taken from the top 0.5 cm volume of dispersion and (if necessary) diluted for further analysis by ICP-OES or ICP-MS methods. The aliquots were taken from the prepared samples every hour, starting hour 0 and finishing hour 6. Such frequency was found to be an optimal time to investigate on dispersibility and dispersion stability in the same time. Before the sampling at hour 6, the samples underwent centrifugation at 1000 rpm for 2 minutes to enhance the particle sedimentation. The particle size cut off of 1 µm was used for the calculations. The aliquots of 0.5 ml were taken from the top layer of sample (40 ml) but not directly at the air/water interface and not deeper than 1 cm.

This volume was diluted into the mixture containing 0.2 ml of HNO_3 and 9.3 ml of MQ water, thus giving a total volume of 10 ml (dilution factor 20). Resulted samples were vigorously shaken by hand after preparation and right before the analysis, to ensure their proper homogenization. Prepared samples were analyzed within 24 hours from the time of preparation. If the NPs are not dissolved by the acid addition, there is a great chance they will agglomerate, settle or/and attach to the tube walls. Erroneous analysis is the outcome. For materials other than under investigation here, the best practice should be developed for each particle type. If analyzing dispersed NPs, methods of slurry analysis should be taken into account. Large particles in the dilute dispersions will increase the relative standard deviation (RSD), might be selectively rejected in the spray chamber, might not be ionized completely, produce higher signal RSD and large agglomerates might clog the nebulizers.

2.1.10 Time resolved sample analysis by UV-VIS

Continuous monitoring of the settling process was performed by means of UV-VIS spectrometry. Samples for analysis of dispersion behavior by UV-VIS were prepared in the way as for ICP-OES/MS measurements regards to sample composition. Samples of interest were analyzed against blank samples in a double-beam photometer. Blank samples were prepared to the same conditions as the samples of interest, but in absence of the investigated nanomaterials. As UV-VIS allows analysis of only one sample at a time, the analysis of only one experimental factor (either electrolyte concentration or DOM presence or pH variation) at a time is possible. Thereby, the preparation of only one sample and one corresponding blank sample at a time is required. All samples were prepared directly before UV-VIS measurements to avoid particle agglomeration followed by enhanced sedimentation before analysis starts. Prior to the measurements the baseline of UV-VIS spectrometer was set to zero by running the blank samples against each other. After this, the sample of interest was inserted to the device and analyzed against the blank sample for 24h, a single measurement being performed each 30 min. The analysis of dispersion was performed ~1.5 cm from the bottom of the UV-VIS cuvette. This is also one reason for longer experimental durations as the measurement takes place at the bottom of the vial in contrast to the former approach, were it is taken at the top.

3 Results and discussion

3.1 Ultrasonic probe experiments: determination of delivered acoustic energy by calorimetric measurements

When preparing the dispersion of analyzed particles, the sonication of dispersion is normally required to provide the homogenous distribution of particles in the dispersion. To obtain reproducible sample preparation it is important to standardize the amount of energy that is transferred into the dispersion. Some instruments display the energy transmitted, but experiments have shown that this is not the value that is received by the dispersion. Hence prior to the dispersion, the delivered acoustic energy was determined by calorimetric measurements.

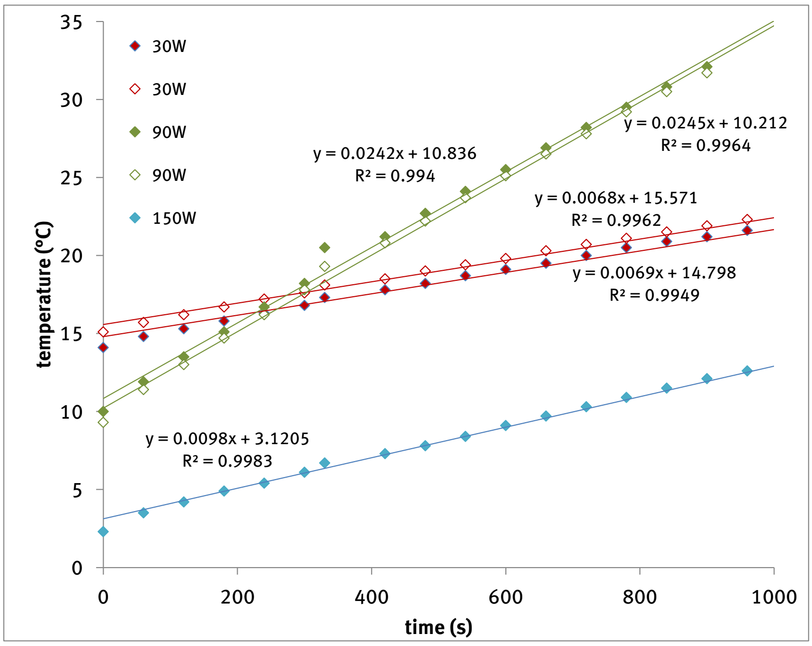


Figure 7: Calibration of delivered acoustic energy during sonication. Determination of slope of the linear trend line fit.

The increase of temperature during the application of ultrasonic energy to 500ml of water by probe sonication was measured and the delivered energy then calculated by evaluation of the slope of the temperature curve, using the formula:

$$E = \frac{dT}{dt} m C_p$$

Where $\frac{dT}{dt}$ is the change of temperature of the media during certain time interval, C_p is specific heat capacity of the medium (4.184 kJ/kg*K for water), m is mass of the medium (kg). The value of $\frac{dT}{dt}$ can be directly found from of the equation of the linear trend line data fit presented in figure 7. The linear trend line has the general equation of y=kx+b, where k = $\frac{dT}{dt}$ and corresponds to the slope of the line. Of course to determine the energy transmitted to the particles, also the volume of the dispersion media needs to be taken into account. Energy should be given as E/V. Results for the experiments shown in figure 6 are calculated and listed in table 1.

Table 1: Comparison of instrument settings, instrument displayed energies and energies received by the sample medium.

power setting on instrument	slope	power measured [W]	energy input measured after 1000s [kJ]	energy displayed [kJ]	% of expected value
30 W	0.0069	14.4	14.4	29.9	48
90 W	0.0242	50.6	50.6	89.7	56
150 W	0.0093	19.5	19.5	67.1	29

The experiments have been conducted in open glass beakers, hence there is a heat transfer from the test vessel to the surrounding air and this energy loss will reduce the measured energy compared to the actually transmitted energy by the instrument. This effect is visible by the slight curvature of the temperature over time graph. The higher the temperature difference to the surrounding air, the higher is the energy loss from the vessel. However, especially at temperatures close to the surrounding air temperature, this loss is negligible. Instead of working in thermally insulated vessels, what might complicate the experimental set-up, it is suggested to work from 5 degrees below to 5 degrees above the ambient temperature. This should give very realistic values.

3.2 Investigations of nanomaterial detection limits of various techniques

The concentrations of the nanoparticles in the topmost part of the vial need to be determined. The techniques generally available for this purpose will report the mass concentrations of the nanoparticles. Since the start mass concentration will be calculated to meet a narrow band of particle number concentrations, the start concentration in mass will vary depending on average particle size and density. At the same time, the final concentration of the test should also be reported, what will require techniques, which are able to measure down to approx. 1 % of the starting concentration. It is assumed that the technique employed should hence be able to quantify 10 µg/L of the nanoparticles safely (limit of detection). Depending on the nature of the nanoparticles, different methods will be suitable for this purpose. It is out of the scope of this study to evaluate all suitable techniques for all possible nanoparticle types. We here concentrate on metal-based particles (accessible by ICP-MS and ICP-OES techniques) and particles that sufficiently scatter light or possess optical properties which enable the use of light absorption. ICP-MS techniques will offer limits of detection far below 10 µg/L for almost all metal-based nanoparticles and will therefore not be investigated in detail. However these instruments are costly and may be replaced by wider available and less costly Nephelometry or UV-VIS spectrometry. These methods also offer the detection of carbon-based materials not directly accessible by ICP-MS or –OES. However, these instruments do not offer the required sensitivity and are not as selective as ICP-MS and might therefore be interfered by e.g. the addition of NOM. It has to be stated that when particle concentrations are determined by ICP techniques and the particles tend to dissolve or the element used for particle analysis is also present in the NOM material used, this has to be taken into account when interpreting the results and by measuring sample blanks and/or the dissolved fraction.

When performing nephelometric or UV-VIS spectrometric turbidity measurements to determine particle concentrations the detection principles have to be taken into account. Nephelometric turbidity measurements are based on the scattering of light by the particles of an incident beam and the scattering intensity is recorded at 90° (or other values ≠ 0°, depending on manufacturer) to the incident beam. Only the scattered light is recorded and therefore light absorbing properties of the medium or the particles play a minor role in the measurements. Hence the measurement is relatively robust to the optical properties of the medium and the measurements are sensitive since the comparably small intensity of the scattered light is not compromised by the high intensity of the incident beam (high amplification of the signal becomes possible, as in e.g. fluorescence techniques). If a UV-VIS photometer is employed, the attenuation of the incident beam (by the scattering of light from the smaller particles or by obstruction by larger particles) is related to the concentration of the particles. If the particles only scatter light (no specific light absorption by the particles) this attenuation is relatively small compared to truly light absorbing substances and the total intensity of the incident light beam, hence the sensitivity is typically less than that from nephelometric measurements. The scattering of light by the particles scales with λ^{-4}, so it is preferable to measure at small wavelengths. However, at small wavelengths and due to the fact that measurements take place on-axis of the incident beam, the contribution of true light absorption by even traces of NOM or other light absorbing components (e.g. nitrate) might overwhelm the signal obtained from the particles' scattering. The results are therefore prone to interferences from the medium and the sensitivity for particles might be diminished by the medium. The magnitude of the signal obtained in both techniques is additionally to the particle mass concentration also depending on the particle size, refractive index and partially even particle shape. Hence a general limit of detection cannot be given, smaller, low refractive index materials will have higher LoDs. A thorough analysis of pros and cons of off-axis nephelopmetric and on-axis UV-VIS spectrometric turbidity measurements is given in (von der Kammer, et al. 2005).

The situation changes for nanoparticles showing true light absorption in a wavelength range not overlapping with the NOM. NM105, as a UV-filter, shows light absorption below λ=400 nm with a maximum at around λ=300 nm. NM300K as a plasmonic metal nanoparticle shows light absorption in the plasmonic region λ=430 nm. While the NM105 spectrum overlaps with the NOM spectrum, the NM300K largely profits from its optical properties for a sensitive detection in on-axis UV-VIS measurements.

3.2.1 Investigation of nanomaterial detection limits of Turbidity (Nephelometry)

Nephelometric turbidity measurements of TiO_2 (NM105) nanoparticles were performed in aqueous solutions, optionally containing 30 ppm DOC (SRNOM) and in absence of salt. The concentration of TiO_2 (NM105) nanoparticles ranged from 1 ppb to 50 ppm. Obtained results for dispersions in MQ water are presented in figures 8a, b.

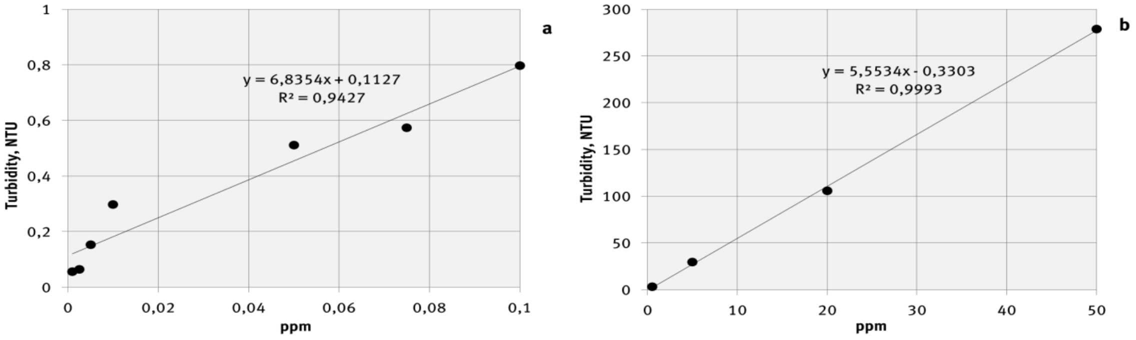


Figure 8: Turbidity measurements of TiO_2 (NM-105) nanoparticles, dispersed in MQ water, in the concentration range of (a) 1-100 ppb, (b) 0.5-50 ppm

Owing to the detection principle (HACH 2100 AN Turbidity Meter according to ISO) the influence of NOM is limited since the light absorption of the NOM is minimal at the wavelength applied for the measurements (λ=870 nm) and the detection is in true scattering mode at 90° angle to the incident light beam. As can be seen from the figures the nanoparticle quantification for the NM105 sample is straight forward above 0.5 ppm and becomes difficult below 0.05 ppm.

Similar results were obtained for the TiO_2 (NM105) nanoparticles dispersed in 30 ppm DOC (SRNOM) containing media. Obtained results can be observed in figures 9a, b.

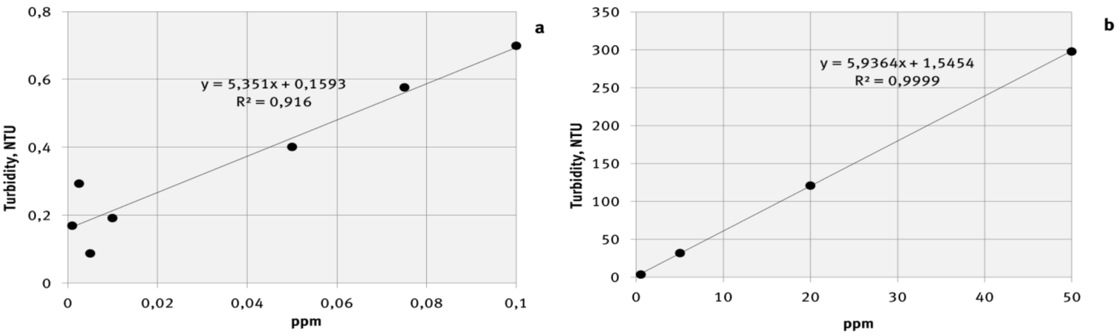


Figure 9: Turbidity measurements of TiO_2 (NM-105) nanoparticles, dispersed in 30 ppm DOC (SRNOM) in the concentration range of (a) 1-100 ppb, (b) 0.5-50 ppm

In case of TiO_2 (NM105) nanoparticles dispersed in 30 ppm DOC (SRNOM) media, the quantification range is similar to the non-NOM sample and corresponded to a TiO_2 (NM105) concentration of 0.05 ppm.

Turbidity measurements of Ag (NM300K) nanoparticles were performed in aqueous solutions, optionally containing 30 ppm DOC (SRNOM) and in absence of salt. The concentration of Ag nanoparticles ranged from 1 ppb to 50 ppm. Obtained results for dispersions in MQ water are presented in figures 10a, b.

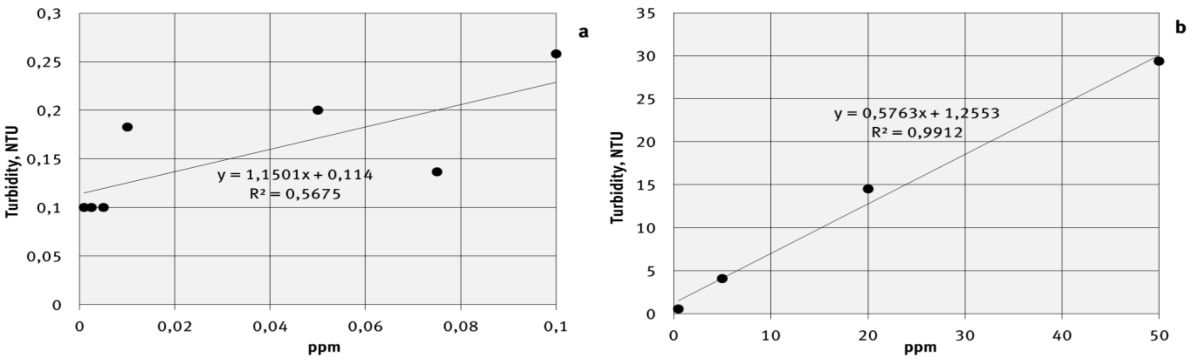


Figure 10: Turbidity measurements of Ag (NM300K) nanoparticles, dispersed in MQ water, (a) in the concentration range of 1-100 ppb, (b) in the concentration range of 0.5-50 ppm

In case of dispersions obtained in MQ water (figure 10a), the background off-set of the nephelometer is 0.1 NTU. The minimal measured concentration of Ag (NM300K) nanoparticles is therefore 10 ppb (0.01ppm). In case of dispersions obtained in 30 ppm DOC (SRNOM), the background off-set of the nephelometer is 0.2 NTU, corresponding to a minimal measured concentration of 0.1 ppm as can be observed in figure 11a, b.

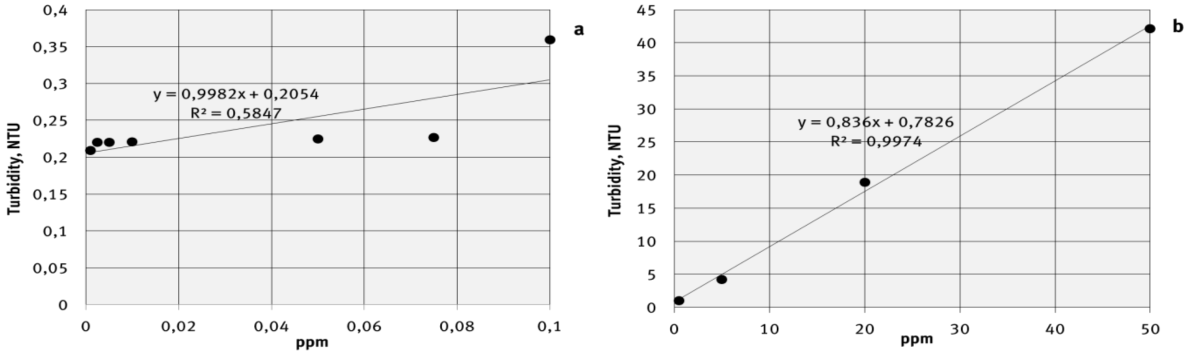


Figure 11: Turbidity measurements of Ag (NM300K) nanoparticles, dispersed in 30 ppm DOC (SRNOM), (a) in the concentration range of 1-100 ppb, (b) in the concentration range of 0.5-50 ppm

3.2.2 Investigation of nanomaterial detection limits by Turbiscan

Formulaction Turbiscan measurements were performed on the dispersions of TiO_2 (NM105) nanoparticles. Dispersions of TiO_2 (NM-105) nanoparticles were prepared in MQ water, 1 mM and 10 mM solutions of $Ca(NO_3)_2$, and in 10 mM $Ca(NO_3)_2$ solution, containing 30 ppm DOC (SRNOM). The samples of 20 ml were analyzed for 6 hours. Calculated rates of sedimentation can be observed in table 2.

Table 2: Calculated rates of sedimentation in dependence of TiO_2 (NM-105) nanoparticles concentration in the dispersion, concentration of $Ca(NO_3)_2$ and presence of 30 ppm DOC (SRNOM)

Sample TiO_2, (NM-105)	sedimentation (mm/h)
10 ppm, MQ	0.00
10 ppm, 1mM $Ca(NO_3)_2$	0.10
10 ppm, 10mM $Ca(NO_3)_2$	0.00
10 ppm, 10mM $Ca(NO_3)_2$, NOM	0.00
30 ppm, MQ	0,00
30 ppm, 1mM $Ca(NO_3)_2$	52.12
30 ppm, 10mM $Ca(NO_3)_2$	90.92
30 ppm, 10mM $Ca(NO_3)_2$, NOM	136.83
50 ppm, MQ	0.00
50 ppm, 1mM $Ca(NO_3)_2$	0.00
50 ppm, 10mM $Ca(NO_3)_2$	76.47
50 ppm, 10mM $Ca(NO_3)_2$, NOM	167.03
100 ppm, MQ	46.15
100 ppm, 1mM $Ca(NO_3)_2$	95.88
100 ppm, 10mM $Ca(NO_3)_2$	152.47
100 ppm, 10mM $Ca(NO_3)_2$, NOM	47.77

Obtained results show that sedimentation of TiO_2 (NM105) nanomaterial can only be investigated by the Turbiscan technique when the concentration of TiO_2 (NM105) in the dispersion is 30 ppm or more. As it was expected, the systems containing the largest amount of electrolyte showed fastest sedimentations of particles as can be seen based on the obtained values of sedimentation rates. It can also be seen that addition of NOM has the expected influence (reducing agglomeration and settling) only for the 100 ppm sample, but not for the 30 and 50 ppm sample.

Turbiscan measurements were also performed on Ag (NM300K) nanoparticle dispersions. Dispersions of Ag (NM300K) nanoparticles were prepared in the same conditions as dispersions of TiO_2 (NM105) nanoparticles, described above. The results of Turbiscan measurements for Ag (NM300K) nanoparticles showed the dispersions of Ag nanoparticles to be completely stable during the entire period of measurements (6h) (data not shown). No rate of sedimentation could therefore be calculated. Sedimentation curves derived from Turbiscan measurements for both materials can be observed in the annex devoted to Turbiscan measurements.

3.2.3 Investigations of nanomaterial detection limits of UV-VIS

Concentration of TiO_2 (NM105) nanoparticles was measured by UV-VIS in aqueous samples with or without DOM (SRNOM), (30 ppm DOC). The concentration of particles in samples varied between 10 ppb - 50 ppm. None of the samples contained $Ca(NO_3)_2$ electrolyte, since the latter would promote the particle sedimentation, what, in turn, would complicate the particle detection by UV-VIS. Prior to the measurements the baseline was established using the media of interest, ensuring that only the TiO_2 particles were detected. Obtained results can be observed in figure 12a, b.

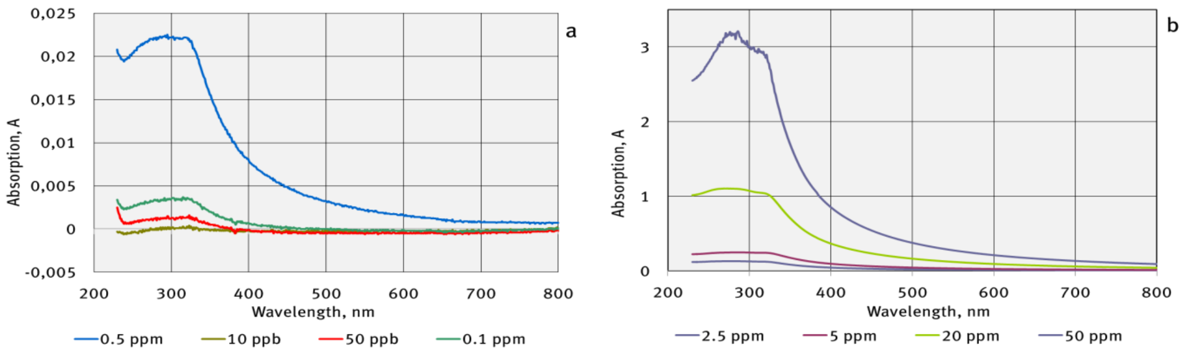


Figure 12: UV-VIS spectra obtained while testing the dispersions of TiO_2 (NM105) with concentrations of (a) 0.01-0.5 ppm and (b) 2.5-50 ppm

According to the obtained spectra, the calibrations lines were obtained as shown on the figure 13a, b. As can be seen from obtained calibration curves the detection limit of 10 ppb is a reasonable limit for UV-VIS technique. The optimal detection wavelength of 300 nm was chosen to build the calibration curves.

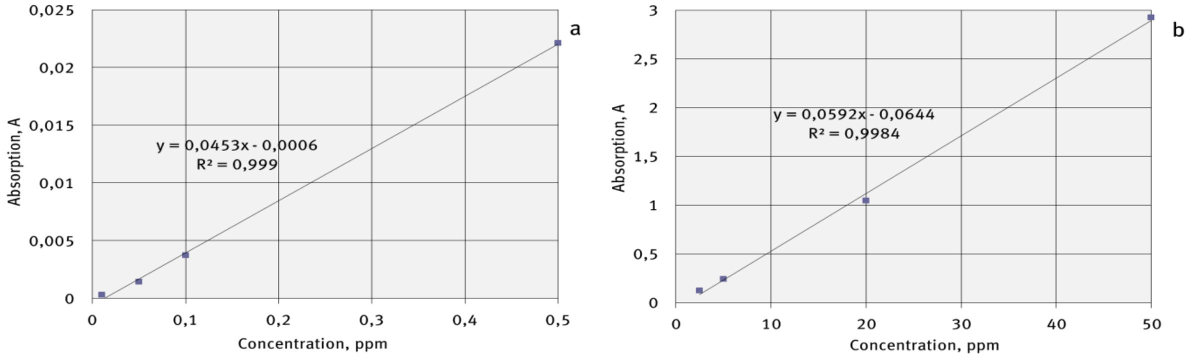


Figure 13: Calibration functions obtained based on the UV-VIS spectra. (a) 0.01-0.5 ppm and (b) 2.5-50 ppm of TiO_2 (NM105) in MQ (without DOC). At optimal detection wavelength of 300 nm

Similar experiments were obtained for dispersions containing TiO_2 (NM105) in 30 ppm solution of DOC (SRNOM). Obtained spectra and corresponding calibration line is presented in figure 14a, b. The detection limit found in NOM containing systems was found to be 0.5 ppm of TiO_2 (NM105), while in MQ based system (figure 13) it was found to be 10 ppb. The optimal wavelength of 300 nm was chosen to build the calibration plot. The absorption spectra clearly show the influence of NOM light absorption below 400 nm, although the measurements were performed against a background solution containing the same amount of NOM.

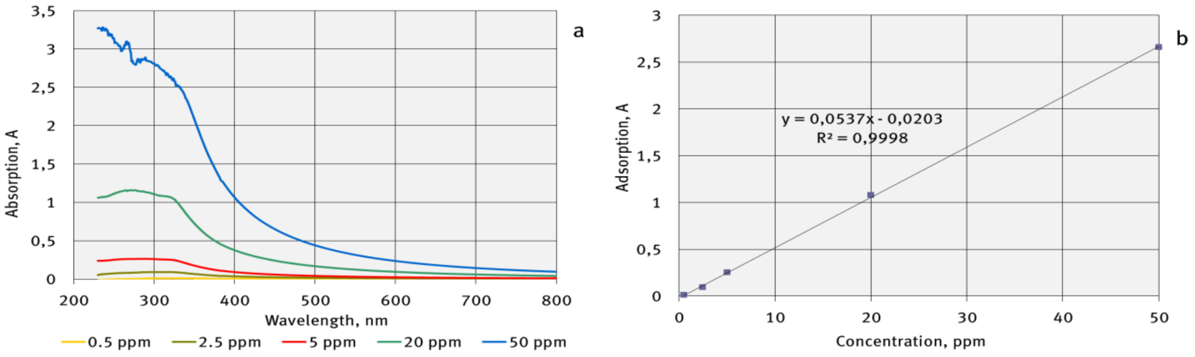


Figure 14: UV-VIS spectra obtained while testing the dispersions of TiO_2 (NM105) with concentrations (a) 0.5-50 ppm in presence of 30 ppm DOC and (b) calibration line based on obtained spectra, (optimal wavelength of 300nm)

Detection limits of Ag (NM300K) nanoparticles were also investigated on aqueous samples with or without DOC (SRNOM), (30 ppm). The concentration of particles in samples varied 5 ppb-20 ppm. None of the samples contained $Ca(NO_3)_2$ salt, since the latter could promote the particle sedimentation, what in turn would complicate the particle detection by UV-VIS. Prior to the measurements the baseline was established using the media of interest, ensuring that only the Ag particles were detected. The results of UV-VIS measurements obtained during analysis of Ag (NM300K)-containing, MQ-based dispersions can be observed at figure 15a, b. Detected optimal wavelength of 430 nm was chosen.

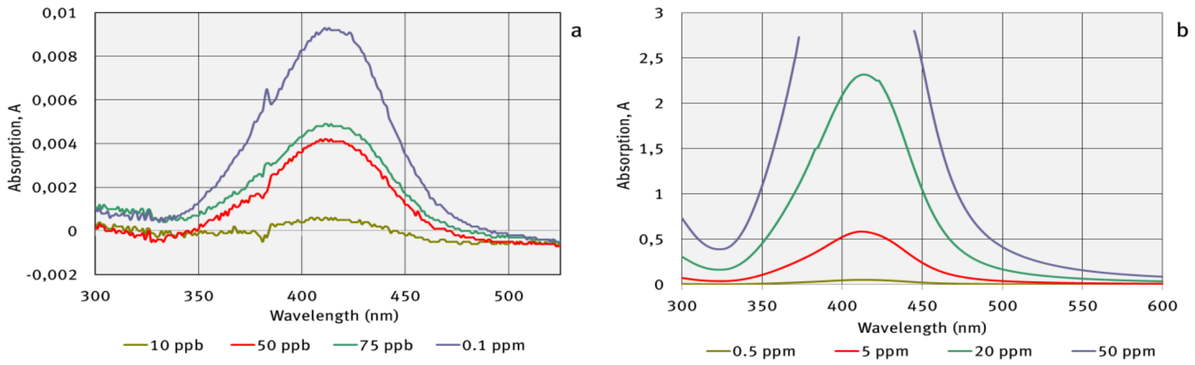


Figure 15: UV-VIS spectra obtained from the dispersions of Ag (NM300K) with concentrations of (a) 0.01-0.1 ppm and (b) 0.5-50 ppm.

Both samples, NM105 and NM300K show true light absorption, NM105 in the UV-range due to its UV-filtering properties, NM300K in the visible light region due to the plasmon resonance. Other particles, as e.g. SiO_2-NPs will not show any true light absorption but only light scattering resulting in turbidity. In turbidity the signal to concentration ratios are much worse compared to true absorption, especially with small particles. Hence for these NPs the detection limits will be inferior to those observed with the two test materials.

Calibration curves obtained based on the UV-VIS spectra of Ag (NM300K) are presented in figure 16a, b. Obtained results thus showed that the detection limits of Ag nanoparticles by UV-VIS method range from 10 ppb to approximately 20 ppm, when particles are dispersed in MQ water.

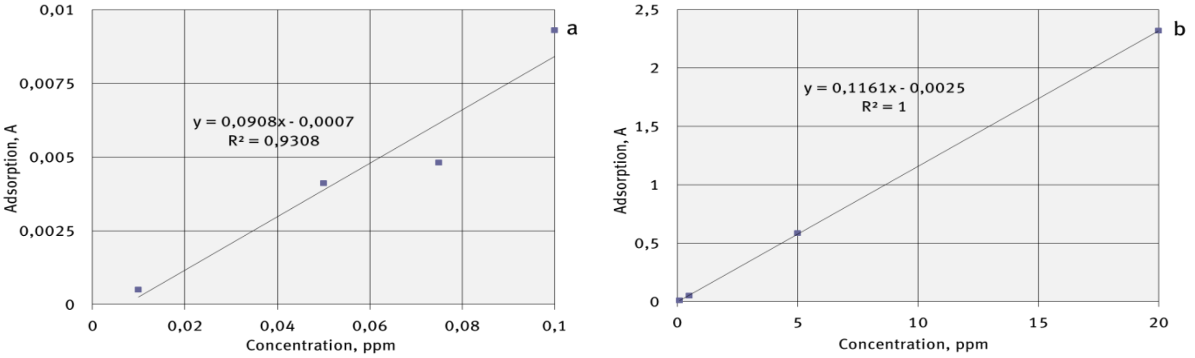


Figure 16: Calibration lines obtained based on the UV-VIS spectra of Ag (NM300K) dispersions. (a) 0.01-0.1 ppm and (b) 0.5-20 ppm of Ag (NM300K) in MQ. Optimal wavelength of 430 nm was used.

Similar experiments were performed on the particles dispersed in 30 ppm solution of DOC (SRNOM). Results are presented in figure 17.

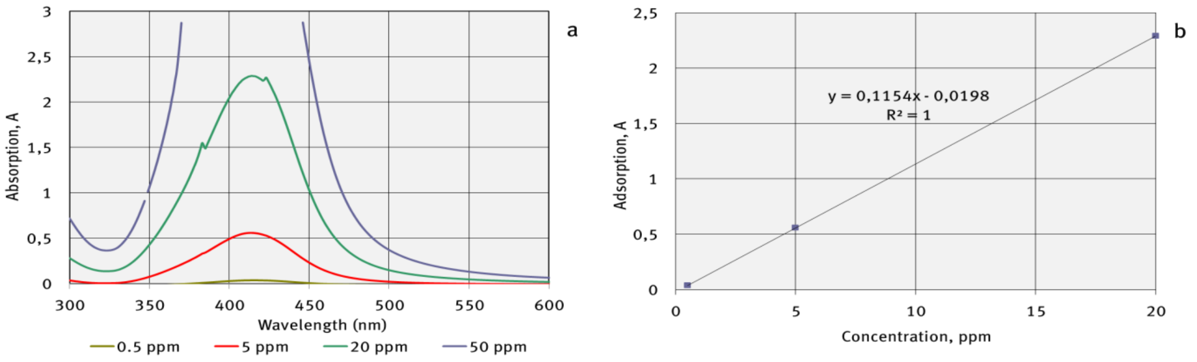


Figure 17: UV-VIS spectra of Ag (NM300K) nanoparticles, dispersed in 30 ppm DOC (SRNOM) solution, (a) with the sample concentrations 0.5-50 ppm (b) calibration curves obtained from the spectra presented at figure (a). Optimal wavelength of 430 nm was used.

Obtained results show that the systems containing SRNOM revealed the Ag (NM300K) particle detection limit of 0.5 ppm. Thus, compared to MQ systems, the sensitivity of method has decreased as was shown before.

3.2.4 Investigations of nanomaterials detection limits of ICP-MS technique

Two dispersions of TiO_2 (NM105) nanoparticles with material concentration of ~1000 ppm were prepared in MQ and 5ppm DOC (SRNOM) solution. These two stock dispersions were diluted 1:20 in MQ and 30 ppm DOC (SRNOM) solutions for further ICP-MS measurements. For each condition four parallel samples were measured. Results of the elemental analysis for total Titanium concentrations are summarized in Table 3.

Table 3: Results of elemental analysis of TiO_2 (NM105) dispersions with different pre-treatments and different dispersion media

Sample	Medium stock dispersion	Medium test dispersion	[TiO_2] (ppb) (+/- 1 SD, N=4)	% of theoretical value (+/- 1 SD, N=4)
TiO_2 MQ 1	MQ	MQ	42.26 +/- 2.56	81.44 +/- 4.94
TiO_2 MQ 2	MQ	30ppm NOM	45.52 +/- 2.17	87.72 +/- 4.18
TiO_2 NOM 1	5ppm NOM	MQ	50.48 +/- 1.48	99.63 +/- 2.92
TiO_2 NOM 2	5ppm NOM	30ppm NOM	55.92 +/-1.94	110.36 +/- 3.83

As can be seen from observed data, recoveries of TiO_2 (NM105) appear at acceptable values (80-110%). Presence of DOM increases the recovery of nanoparticles.

Two dispersions of Ag (NM300K) with a silver concentration of 1 mg L^{-1} were prepared in MQ and 30 ppm DOC (SRNOM) solution. These two stock dispersions were diluted 1:200 in 2% HNO_3 and then analyzed by ICP-MS. The results of the total Ag analysis are summarized in Table 4. Recovery values refer to a theoretical concentration of 5 µg L^{-1}, which is based on the assumption of 10% w/w Ag-concentration in the original NM 300K vials and the dilution factors applied during the preparation of the test dispersions.

Table 4: Results of NM300K AgNPs total analysis by ICP-MS

Sample	Medium test dispersion	[Ag] (ppb) (theoretical)	% of theoretical value (5 ppb)
Ag (NM300K) MQ 1	MQ	4.90	98
Ag (NM300K) MQ 2	MQ	1.45	29
Ag (NM300K) NOM 1	30ppm DOC	4.55	91
Ag (NM300K) NOM 2	30ppm DOC	4.79	96

As can be seen from observed data, recoveries of Ag (NM300K) nanoparticles appear at acceptable values (90-98%), while the sample that revealed the recovery of 29% is a clear outlier. Presence of DOM did not influence the recovery of nanoparticles in this case.

3.3 Time-Resolved investigations of nanomaterial dispersion stability by various techniques

3.3.1 Representation of nanomaterial agglomeration behavior investigated by ICP-OES/MS and UV-VIS measurements

Results obtained during investigations of nanomaterial agglomeration through ICP-OES/MS measurements are presented in the form of plots, where X-axis stays for time of sampling (0 to 6 hours) and Y-axis stays for the percentage (%) of the concentration of analyzed material from the expected concentration (theoretical value, retrieved from weighted amount and volume of sample) of the material in the samples of interest. Expected material concentration was calculated from the amount of material used to prepare the dispersions of interest. The points of mean values belonging for similar dispersions (i.e. either for dispersion containing 1mM $Ca(NO_3)_2$ or for dispersion containing 10 mM $Ca(NO_3)_2$) were presented within one experimental dependency.

In case of investigations of particle agglomeration behavior by UV-VIS the results were presented in the form of plots, where X-axis stays for the absorption wavelength, and Y-axis represents the relative to the initial value absorption (A/A_0).

3.3.2 Agglomeration behavior of TiO_2 (NM105) in presence of electrolyte and DOM in the dispersions

Obtained results are presented in figure 18a, b. As can be seen from obtained results, at no electrolyte addition the NPs are stable over the entire duration, the system containing 1 mM $Ca(NO_3)_2$ reveals particle agglomeration after centrifugation of dispersion at 6h time point. At the same time, addition of DOM to the same media stabilized the 1 mM $Ca(NO_3)_2$ system over entire time of investigations (red lines).

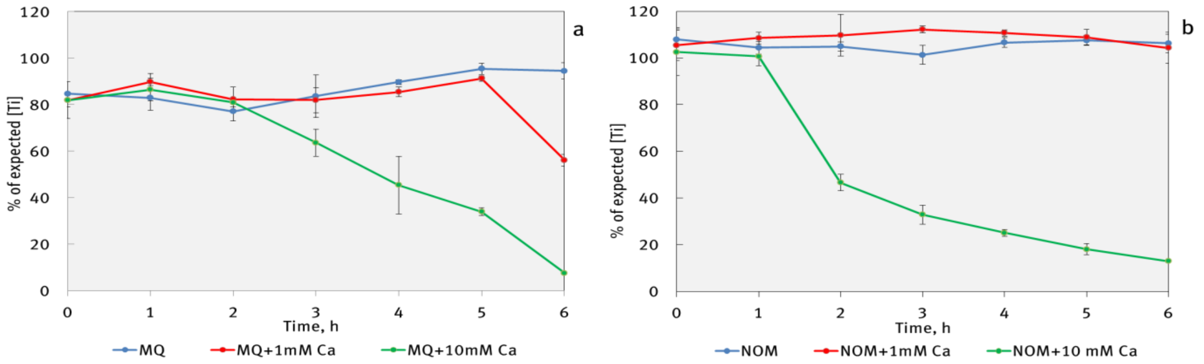


Figure 18: Agglomeration behavior of TiO_2 (NM105) nanoparticles. (a) in 0, 1, 10 mM $Ca(NO_3)_2$ media, (b) in 0, 1, 10 mM $Ca(NO_3)_2$ and 30 ppm DOC (SRNOM) media. Error bars from 3 replicate experiments.

Addition of DOM provides additional stability to the dispersions, through the electrostatic repulsion of DOM molecules adsorbed on the surfaces of investigated nanoparticles. At the same times systems that did not contain any electrolyte were stable over entire period of investigations (blue lines). Systems with 10 mM electrolyte concentration on the other hand showed particle sedimentation with no respect to presence of DOM in the systems. Thus in these systems electrolyte concentration is the factor that overtakes the influence of DOM on particle agglomeration stability. It is also clear that without the addition of DOM there is an apparent loss of material from preparation of the dispersion to the first measurement (curve starts at less than 100%).

3.3.3 Agglomeration behavior of Ag (NM300K) nanoparticles in presence of electrolyte and DOM

Obtained experimental results are presented in figure 19a, b. As can be seen the presence of $Ca(NO_3)_2$ electrolyte in combination or without DOM didn't lead to any particle agglomeration in case of Ag

(NM300K) dispersions. The NM300K NPs are stable under all electrolyte concentrations with and without the addition of DOM.

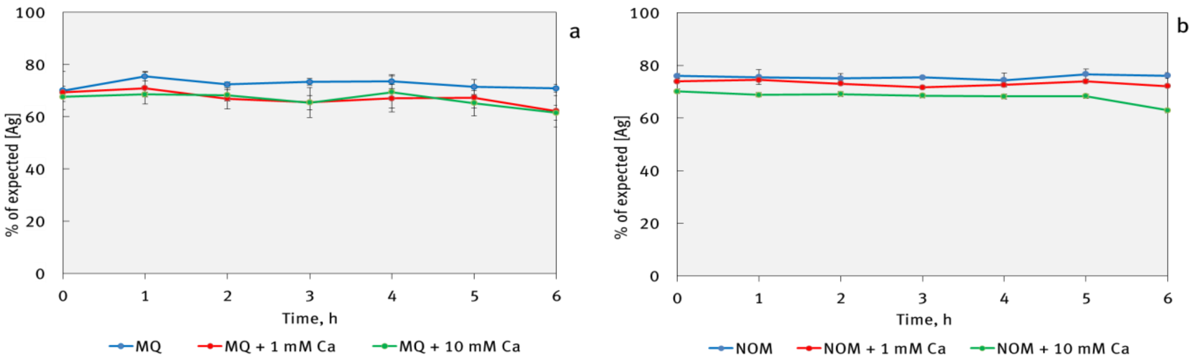


Figure 19: Agglomeration behavior of Ag (NM300K) nanoparticles. (a) in 0, 1, 10 mM $Ca(NO_3)2$ media, (b) in 0, 1, 10 mM $Ca(NO_3)_2$ and 30 ppm DOC (SRNOM) media. Error bars from 3 replicate experiments.

3.3.4 Dependency of TiO_2 (NM105) agglomeration behavior on DOM/DOC concentration.

The used concentration of DOC=30 ppm corresponds to the 95% percentile of DOC concentrations found in surface waters. Since this represents a worst case scenario and due to the high cost of the suggested DOM (IHSS Suwannee River NOM, SWNOM) the concentration in principle can be reduced. Thus in case of TiO_2 (NM105) nanomaterial, DOM revealed the dispersion stabilizing properties down to the concentration of DOC=1.5 ppm (figure 20). Tests were performed on the TiO_2 (NM105) particles, since dispersions of this material were metastable compared to those of Ag (NM300K).

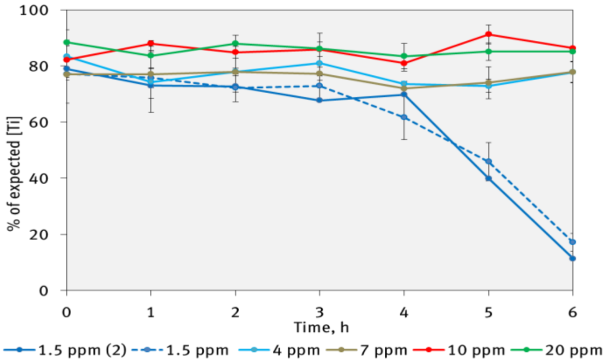


Figure 20: Agglomeration behavior of TiO_2 (NM105) nanoparticles in 1 mM $Ca(NO_3)_2$ and different concentrations of DOC (SRNOM). Error bars from 3 replicate experiments.

In principle the stabilizing effect of DOM can be simulated through addition of any other type of NOM. However, usage of other material besides suggested SRNOM requires careful assessment of the chemical cleanness and source of used material. Special attention shall be paid to the concentration of multivalent cations and anions in the used material, since their presence can lead to the unexpected nanomaterial agglomeration, despite the expected stabilizing effect of DOM, additionally the less purified DOM will show self-agglomeration at low pH or high Ca^{2+}. To illustrate the differences in the contamination of NOM materials, elemental analysis and ion chromatography analysis of SRNOM and Sigma-Aldrich Humic acid (a common replacement of expensive SRNOM) was performed. Obtained results can be observed in table 5 and table 6 respectively.

Table 5: Elemental analysis of Suwannee River NOM and Sigma-Aldrich Humic Acid (HA) (mg of ions per 1g of DOC)

Sample	Metal concentrations as mg/g DOC								
	Ca	Mg	Na	K	Fe	Ni	Cu	Mn	Co
SRNOM1	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_HA1	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 approx. 1g of DOC.)

Table 6: Ion Chromatography analysis of Suwannee River NOM and humic acid (mg of ions per 1g of DOC)

	F^-	Cl^-	NO_2^-	Br^-	NO_3^-	PO_4^{3-}	SO_4^{2-}
SRNOM	n.a.	55.8	n.a.	0.5	35	n.a.	11.3
SigmaHA	2.5	277	n.a.	0.6	20	n.a.	7.8

SRNOM: Suwannee River Natural Organic Matter, HA: humic acid. (Concentrations of ions per approx 1g of DOC)

3.3.5 Agglomeration behavior of TiO_2 (NM105) and Ag (NM300K) nanoparticles in absence of CO_2

Obtained experimental results for dispersions of TiO_2 (NM105) material are presented in figure 21a, b. To confirm the obtained data, experimental results are presented in duplicates (dashed lines). Comparing the presented graphs with the graphs in figure 18, one can conclude, that the absence of CO_2 did not influence the stability of TiO_2 (NM105) nanoparticles neither in MQ media containing electrolyte (figure 21a), nor in media containing DOC and electrolyte (figure 21b).

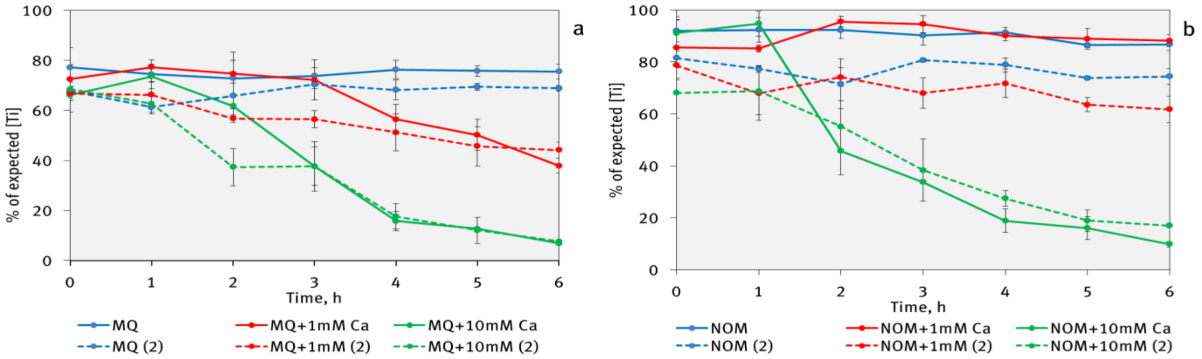


Figure 21: Agglomeration behavior of TiO_2 (NM105) nanoparticles in absence of CO_2. (a) in 0, 1, 10 mM $Ca(NO_3)_2$ media, (b) in 0, 1, 10 mM $Ca(NO_3)_2$ and 30 ppm DOC (SRNOM) media. Replicate experiments marked with “(2)”.

Obtained experimental results for dispersions of Ag (NM300K) material are presented in figure 22a, b. Comparison of presented graphs with graphs in figure 19 shows that absence of CO_2 in the atmosphere surrounding analyzed dispersions did not influence the stability of dispersions containing Ag (NM300K) neither in dispersions with electrolyte nor in dispersions containing DOC.

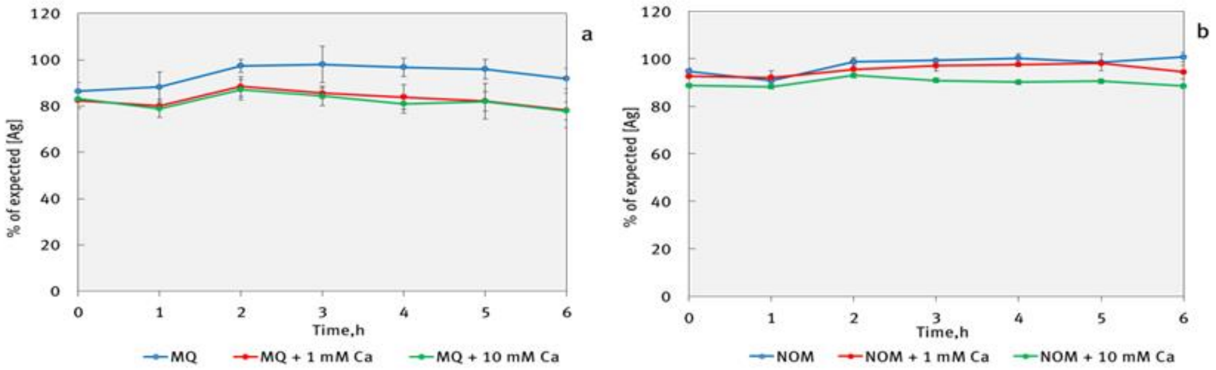


Figure 22: Agglomeration behavior of Ag (NM300K) nanoparticles in absence of CO_2. (a) in 0, 1, 10 mM $Ca(NO_3)_2$ media, (b) in 0, 1, 10 mM $Ca(NO_3)_2$ and 30 ppm DOC (SRNOM) media.

It can be concluded for the tested materials that atmospheric CO_2 intrusion into the test systems and resulting carbonate adsorption on the NPs surfaces does not influence the stability under the chosen conditions. As all higher tier tests will be carried out under normal atmosphere it might not be necessary to account for the effect of CO_2.

3.3.6 Influence of pH on the agglomeration behavior of TiO_2 (NM105) and Ag (NM300K) nanoparticles

Particle dispersion stability at established pH=8.5 was tested, since this pH was the most unstable pH of all investigated (5/7/8.5). Obtained results are presented in figure 23.

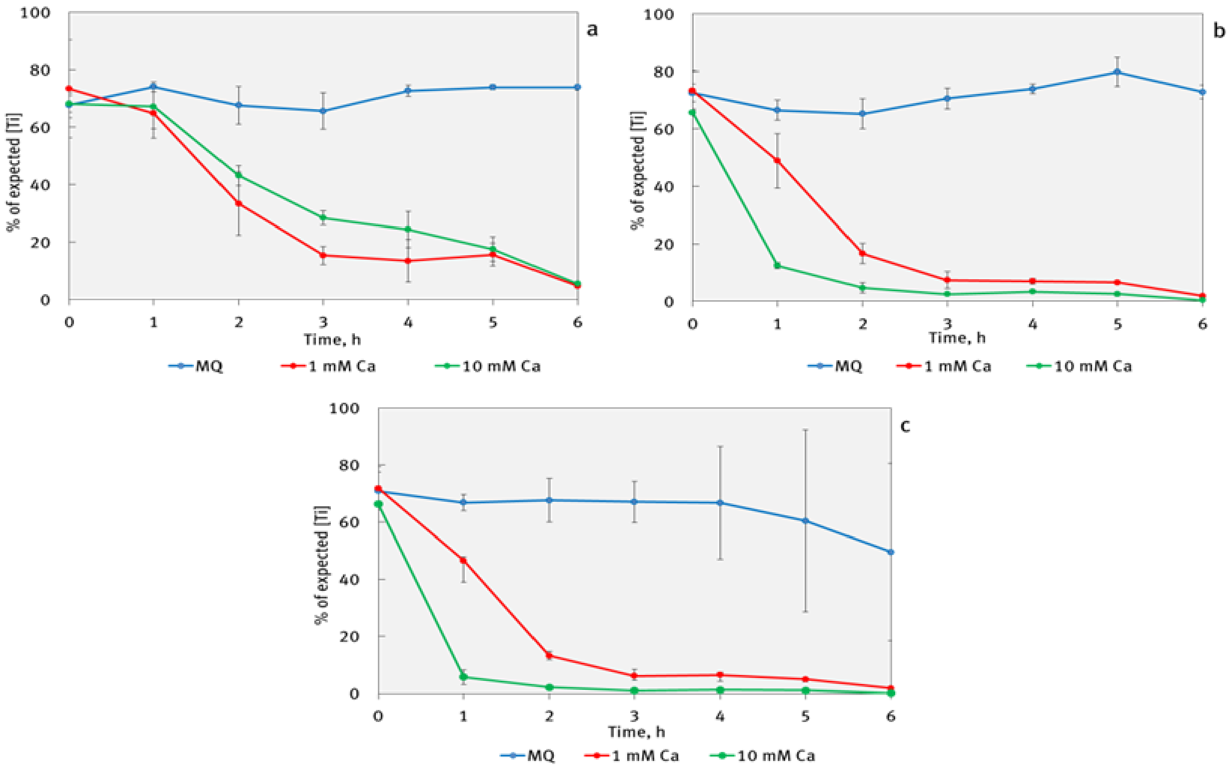


Figure 23: Agglomeration behavior of TiO_2 (NM105) nanoparticles at pH=8.5. pH values were established in presence of (a) 1 mM $NaHCO_3$, (b) 5 mM $NaHCO_3$, (c) 10 mM $NaHCO_3$ and the pH values remained stable during all experimental procedures.

As in previous experiments, establishing the pH of Ag (NM300K) dispersions at the value of 8.5 did not lead to the particle agglomeration as shown in figure 24. Experiments were as well performed in presence of 5 mM $NaHCO_3$, which provided buffering capacity.

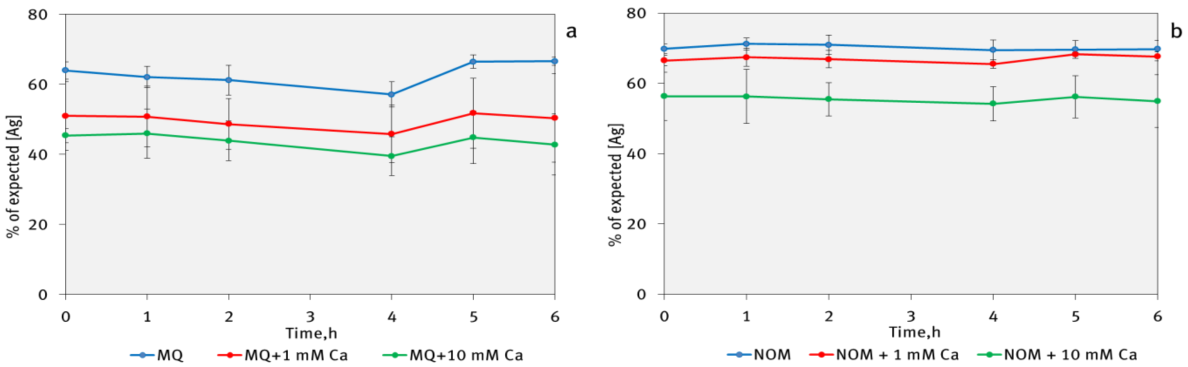


Figure 24: Agglomeration behavior of Ag (NM300K) nanoparticles in dispersion with established pH=8.5. (a) in 0, 1, 10 mM $Ca(NO_3)_2$ media, (b) in 0, 1, 10 mM $Ca(NO_3)_2$ and 30 ppm DOC (SRNOM) media.

3.3.7 Influence of particle concentration on their agglomeration behavior

Agglomeration behavior of TiO_2 (NM105) nanomaterial was tested in dependence of various particle concentrations: at approximately 10^{10}, 10^{11} and 10^{12} particles/L. Experiments revealed that in dispersions with destabilizing Ca-concentrations the agglomeration of dispersions with higher particle number concentrations proceeded faster (figure 25). All experiments were performed in the absence of DOM in the samples.

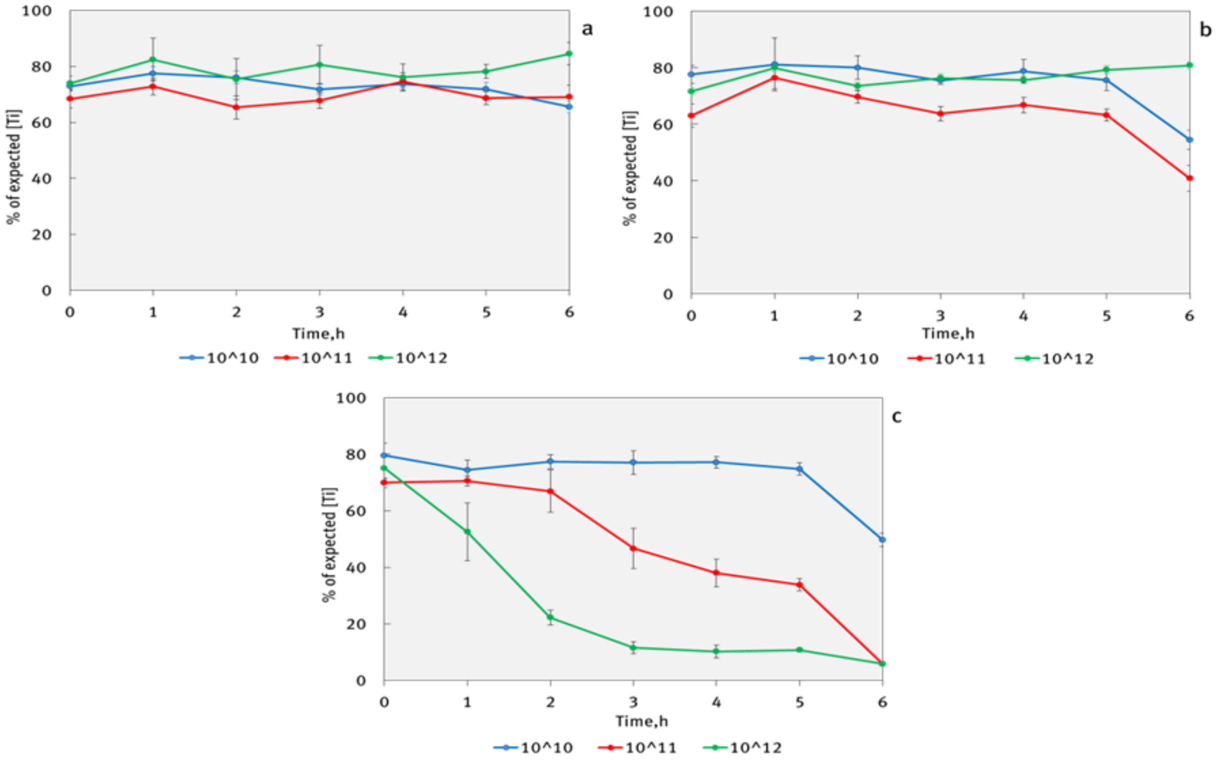


Figure 25: Agglomeration behavior of TiO_2 (NM105) nanoparticles in the dispersions of various particle number concentrations: 10^{10}, 10^{11}, 10^{12} particles /L. (a) in MQ, (b) in 1 mM $Ca(NO_3)_2$ and (c) in 5 mM $Ca(NO_3)_2$.

Figure 25 clearly shows the importance of a defined starting concentration in numbers of particles. At a mildly de-stabilizing condition of 1 mmol/L electrolyte the three different concentrations result in similar plots as would be observed at one concentration and three different electrolyte concentrations

(0, 1, 10 mmol/L), compare with figure 18. Based on the obtained experimental results it can be observed that the optimal particle number concentration to study agglomeration behavior of TiO_2 (NM105) in the described test layout within 6h is 10^{12} particles/L.

Because Ag (NM300K) dispersions revealed high stability during all previous experiments it was decided to test the possibly highest concentration of particles in the dispersions. Thus, the concentrations of 10^{12}, 10^{13}, 10^{14} particles/L were tested. As in previous experiments the dispersions containing different numbers of Ag particles were stable at all conditions. Obtained results are presented in figure 26.

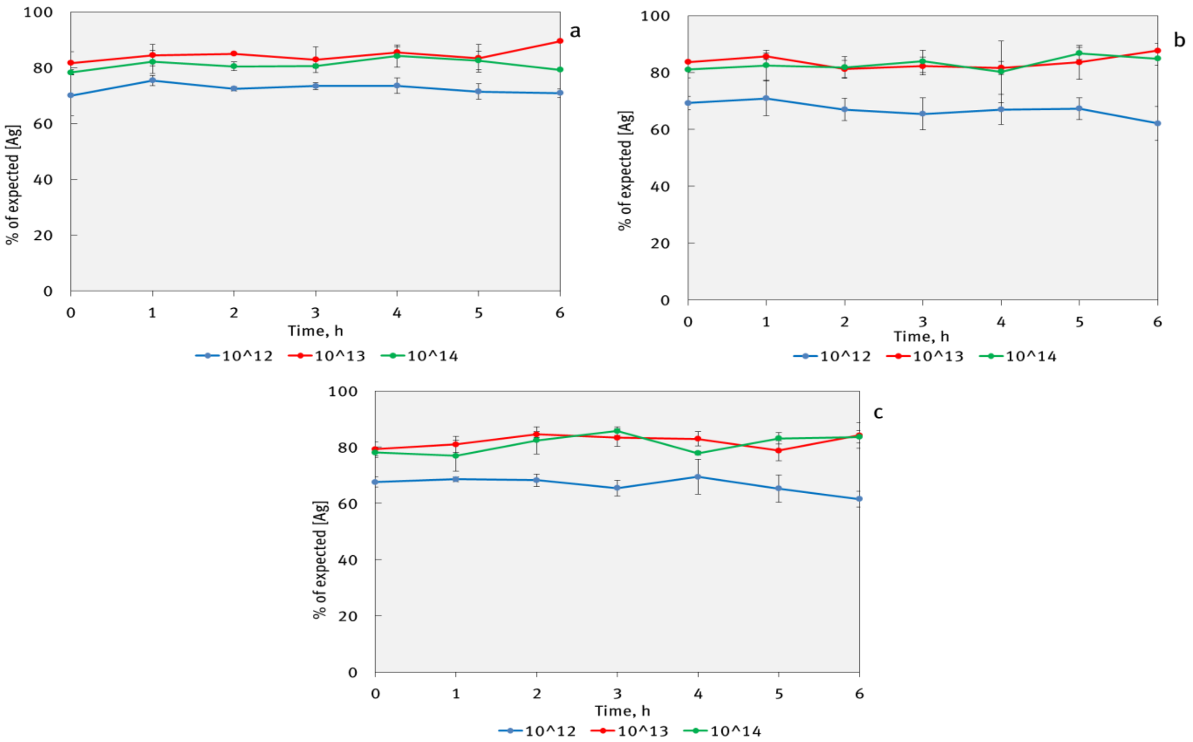


Figure 26: Dispersion stability of Ag (NM300K) particles in the dispersions of various density: 10^{12}, 10^{13}, 10^{14} particles /L. (a) in MQ, (b) in 1 mM $Ca(NO_3)_2$ and (c) in 5 mM $Ca(NO_3)_2$.

3.3.8 Continuous monitoring of nanoparticle agglomeration with UV-VIS spectrophotometers

Agglomeration behavior of TiO_2 (NM105) particles was followed by means of UV-VIS spectroscopy. UV-VIS measurements were performed over time period of 24 hours with the step of 0.5 hour. Such time frame was chosen, since the point of analysis is located at the bottom of the vial, where effects of sedimentation will take longest to become visible. Since it was found earlier that dispersions of Ag (NM300K) particles are stable over entire test period, these dispersions were not investigated by UV-VIS. Obtained results are presented in figures 27a, b for the dispersions in MQ media containing 0, 1 and 10 mM electrolyte and figures 28a, b for dispersions in 30 ppm DOC (SRNOM) media with same concentrations of electrolyte. Obtained dependencies were similar to those obtained through ICP-OES measurements. As can be seen, the optimal wavelengths of 380 nm and 600 nm were chosen in this case to avoid the interactions of TiO_2 with DOM under the continuous UV irradiation.

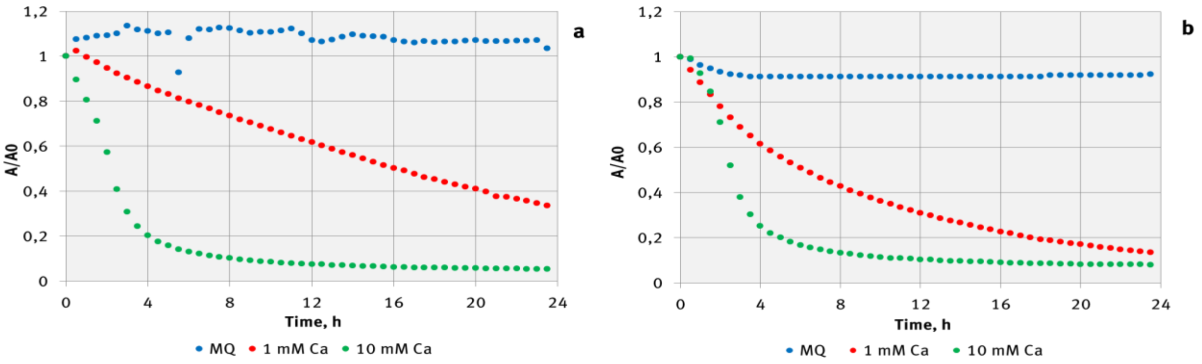


Figure 27: Dispersion stability of TiO_2 particles in MQ media containing 0, 1 and 10 mM electrolyte (blue, red and green lines correspondingly). (a) at 380 nm wavelength. (b) at 600 nm wavelength.

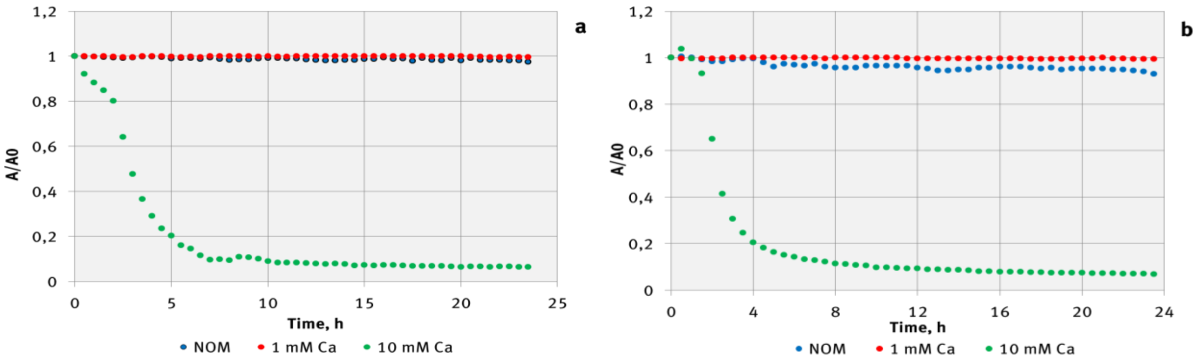


Figure 28: Dispersion stability of TiO_2 particles in solution of 30 ppm DOC (SRNOM) media containing 0, 1 and 10 mM electrolyte (blue, red and green lines correspondingly). (a) at 380 nm wavelength. (b) at 600 nm wavelength.

3.4 Validation of obtained results

In order to validate the results obtained in this research and to evaluate the proposed procedure to test the dispersion behavior of nanomaterials based on these results, an inter-laboratory test was organized in summer 2015. For this aim, a draft Test Guideline was distributed to 12 interested laboratories. Out of those 12, seven laboratories did provide the experimental data. After the data was obtained from the partner labs, statistical analysis was performed in order to check for similar character of obtained dependencies. Reasons for outlying data were identified and subsequently, outlying data weren`t considered in the statistical analysis. During the statistical analysis the experimental points obtained from independent laboratories were gathered and the mean values of all points obtained during similar sampling time were calculated within one experimental dependency, (i.e. either for dispersion containing 0 mM $Ca(NO_3)_2$, 1mM $Ca(NO_3)_2$, or for dispersion containing 10 mM $Ca(NO_3)_2$). After that the standard and the mean deviation values for all the points obtained from independent laboratories were calculated and presented as a result of statistical analysis.

Obtained results have shown that the character of statistically analyzed experimental dependencies is highly similar. Thus, initial expectations for the similarity of obtained experimental results were fulfilled. Based on this finding, the testing procedures proposed in the draft Test Guideline seems valid. Calculated mean and standard deviations remained within 25% and 29% of total material concentration, respectively in case of TiO_2 (NM105) and within 26% and 34% of total material concentration, respectively in case of Ag (NM300K). Such values can be considered as fully acceptable providing the fact that the losses of material due to experimental manipulations stay within the magnitude of provided values. Largest standard and mean deviations for each test and corresponding materials are presented in table 7 and table 8.

Table 7: Largest standard and mean deviations for experiments on TiO_2 (NM105)

TiO_2 (NM105)	Max. mean dev. (%)	Max. standard dev.(%)
electrolyte	15.85	21.48
CO_2	10.45	14.32
NOM	25.11	29.32
pH	18.47	24.37

Table 8: Largest standard and mean deviations for experiments on Ag (NM300K)

Ag (NM300K)	Max. mean dev. (%)	Max. standard dev.(%)
electrolyte	26.27	34.25
CO_2	18.38	23.98
NOM	13.89	22.81
pH	10.64	15.05

It was found that to obtain the correct results the analysis of prepared samples shall be performed as soon as possible, at least within 24 hours. In case these conditions could not be fulfilled, full digestion of the samples shall be applied and the digestion procedure should be able to fully dissolve the nanomaterial (e.g. HF/HNO_3 digestion for TiO_2 ENPs, aqua regia for Au-ENPs). Longer storage of the samples led to particle sedimentation and attachment to the walls of the sample vials. This leads to incorrect measurements as was found on the example of TiO_2 (NM105) nanoparticles. Sonication might re-establish a homogeneous distribution of the ENPs in the vial for a short period of time, but detachment of ENPs attached to the walls is unlikely to be complete. Manual manipulations of the samples have to be performed carefully, avoiding unnecessary disturbance, such as placing the samples on the shakers, etc. for significant periods of time. Such conditions will likely lead to the particle agglomeration, settling and attachment to the wall of the sample vials as was demonstrated on the example of Ag (NM300K) particles.

3.5 Development of tiered dispersion behavior scheme

The decision tree, developed as a result of this study, provides the experimental routine to test the dispersion behavior of nanomaterials in aquatic media to estimate their possible fate in and impact on the environment and in higher tier testing. The decision tree is designed to address the issue of nanomaterial dispersion behavior in such way that would require the minimal time- and cost- spending, but allow the complete understanding of reasons and factors influencing this process (Figure 29).

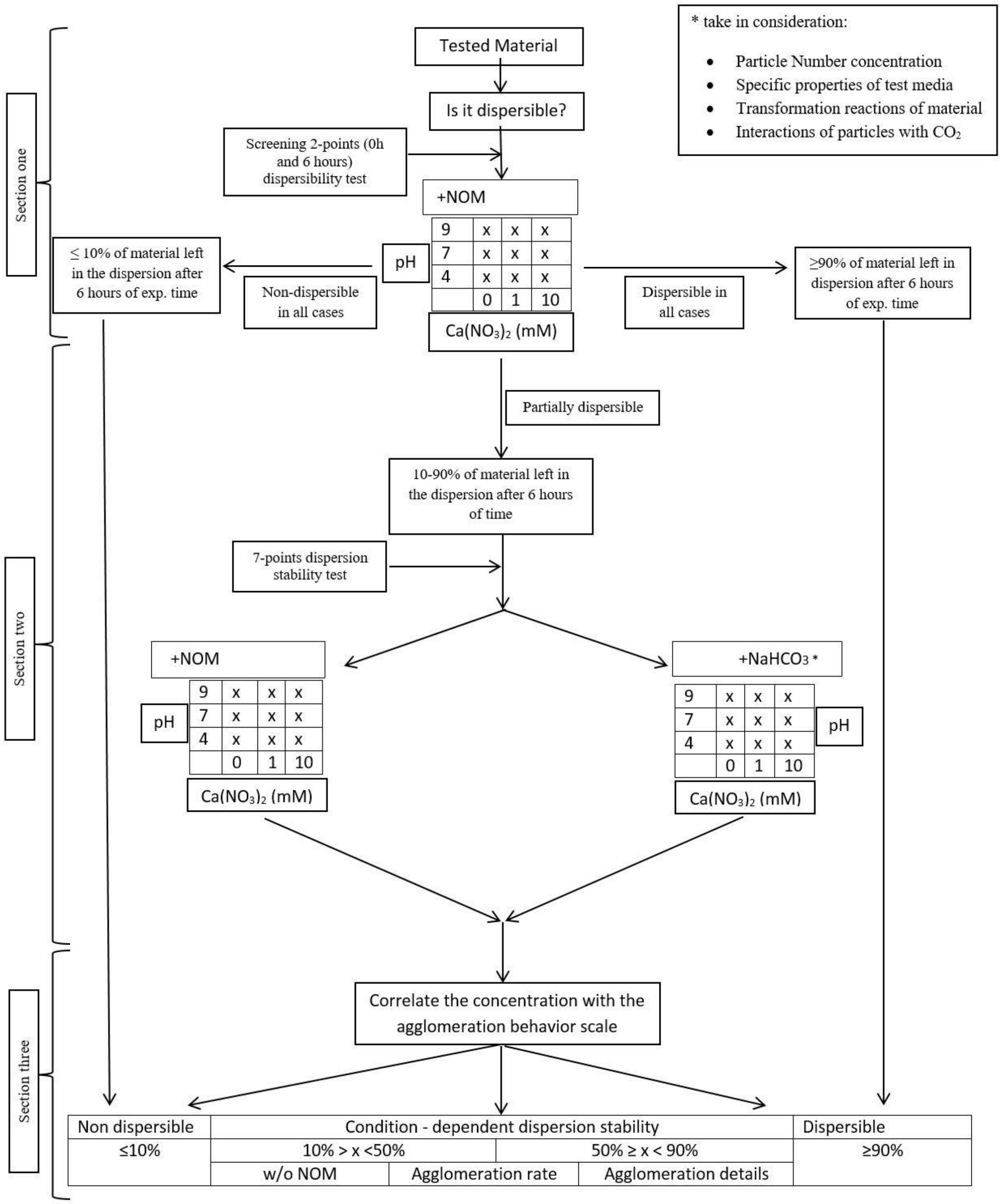


Figure 29: Suggested tiered agglomeration behavior scheme

Depending on the results the investigated nanoparticles can be classified according to the presented decision tree, where class (a) is assigned to non-dispersible materials, class (b) is assigned to materials with condition-dependent dispersion stability and class (c) is assigned to the case of fully dispersible materials. With materials of (a) class remains ≤10% of the initially detected (0 h measurement) material concentration in the form of dispersion after 6 hours of experimental time in presence of factors responsible for agglomeration (pH=4, 7, 9; $[C]_{electrolyte}$=0,1,10 mM, presence of DOM). Materials that remain 10-90% of material in the form of dispersion after 6 hours of experimental time and applied conditions are related to class (b). Finally, class (c) is prescribed to the materials that remained ≥90% of initially detected material after similar experimental time and applied conditions.

Section one of the decision tree suggests to refer the analyzed material to one of the mentioned classes (a), (b), or (c) by determination of material dispersibility through 2-points test (0, 6 h). The 2-point dispersibility testing is sufficient for testing of class (a) and class (c) materials. Contrary, (b) class material would require the full (7-point = 0,1,2,3,4,5,6 h) dispersion stability test in all conditions, including and excluding presence of DOM (Section 2 of decision tree). After 7-point dispersion stability test obtained results can be related to the scale of dispersion stability (Section 3 of decision tree). Obtained material concentration can stay within the range of 10-50% or 50-90% of initial measured concentration. The time-dependent agglomeration behavior in the 7-point testing reveals information about the agglomeration process. This shall be discussed taking into account the hydrochemical conditions and (if available) additional information about the nanomaterial. When working with the materials that reveal condition-dependent dispersion stability one shall keep special attention to the smallest variation of factors influencing agglomeration behavior of nanomaterials.

3.6 Development of a Guidance Document

Within this project, a first internal draft Guidance Document (GD) was developed for both, the TG on dispersion behavior and the (still under development) TG on dissolution rate of nanomaterials for investigation in simulated environmental media. The GD should serve as a support tool for both the TGs, to provide guidance on the influence of various experimental conditions and materials on the procedures of the TGs, as well to inform on the applicability of these procedures to the various nanomaterials and give support for the interpretation of results. This draft GD in the current state only addresses the TG on dispersion behavior since it is the most advanced of both TGs which shall be covered by the GD. The internal draft GD discusses the relevance and applicability (including limitations) of the suggested experimental approach, includes a section on the evaluation of experimental factors which are influencing the behavior of nanomaterials in the aqueous media, describes the application of the decision tree approach including the decision tree threshold values and supports the interpretation of the results towards the further fate and eco-toxicological testing.

The round robin testing of the TG and the commenting rounds in the OECD test guideline program (WNT) additionally pinpointed specific materials, experimental steps and procedures which need to be covered in the GD. Among those are:

- the interconnection between the defined particle number concentration at the beginning and the test duration of 6 hours

- the type and quality of the used NOM and the problematic use of other, less pure NOM or the use of user-specific, possibly locally acquired, NOM

- the meaning of using synthetic media compared to using local (filtered) lake or river water

- the transferability to test eco-toxicological test media via pH values and ionic composition

The development of the GD is still ongoing. The draft GD developed within this project serves as input document for the further development of the GD in the framework of the OECD WNT. Therefore, the draft GD is not published with this report.

4 Overall conclusions

It could be shown on three exemplary materials with varying abilities to produce stable aqueous dispersions that the suggested testing scheme provides an operationally defined scheme to compare ENPs for their dispersion behavior. Additionally the test gains information on the dispersion stability and agglomeration behavior of ENPs under a wide range of conditions representative for the agglomeration potential of natural waters. Taking the pH and divalent ion concentration of eco-toxicological test media into account predictions on ENP behavior in test systems are possible. The test procedure is designed to produce reproducible test results and the round robin testing has shown that it can be reliably executed in different laboratories.

The outcome of the experiments described in this report were included in the draft OECD Test Guideline on Dispersion stability of Nanomaterials in Simulated Environmental Media. The first draft was submitted to the OECD WNT in December 2015 followed by the WNT commenting rounds in 2016 and 2017. It was approved by WNT in April 2017.

In the course of the discussions in the OECD expert group associated with this development and also during the commenting in the OECD WNT it became apparent that several conflicts of objectives exist. The most important one seems to be the balancing of experimental effort and required expert knowledge with the highest possible realism of the testing procedure to represent most naturally occurring processes, an everlasting conflict in the environmental sciences in general.

The test looks only at homoagglomeration while in natural systems we would expect heteroagglomeration with natural particles to be the fate-dominating reaction. The reason not to develop a heteroglomeration test laid in the fact that such experimental routines are still little developed and the necessary analytical efforts would be by far greater. Another reason is that homoagglomeration might represent a worst case in stability, because any introduction in heterogeneity (difference in particle size, “patchy” surface charge, shape) on the side of the natural particle as counterpart will most likely reduce the stability of the dispersion. This however, although appearing somehow logic, still needs to be proven. Another obstacle that comes with a test employing heteroagglomeration is the type and origin of the natural counter-particle that should be used in the heteroagglomeration test. The test aims to compare nanomaterials with each other and this would require a standardized and reproducibly dispersed natural counter-particle (e.g. suspended particulate matter) which is representative for natural processes. This counter-particle does not exist, neither conceptually nor in practice.

The selection of water composition and pH misses out many of the cations and anions present in natural waters. That the chosen parameters and components are a reasonable simplification of the complex natural environment has been laid out in the report, however, it was assumed that effects from other components of the same character (valency, charge, DOC) add in a linear way. If there are non-linear synergistic or antagonistic effects, this has not been investigated yet. However, within two papers, Liu et al. (2013) and Hammes et al. (2013) used the same dataset (FOREGS), but a completely different approach to estimate the influence of surface water chemistry on dispersion stability and independently came to very much the same conclusions. While Lui et al. (2013) used stability values derived from a set of stability testing as described here, using each a single component (NaCl, $CaCl_2$, DOM and pH) and calculated the dispersion stability of Gold-NPs across Europe according to the local water chemistry in 808 surface water samples across Europe, Hammes et al. (2013) did a multivariate analysis of the complex water chemistry data to arrive at 6 different major water chemistries in different regions of Europe. These of course remained still complex as they contained all components for which data was available. They then calculated theoretical thicknesses of the double layer to derive nanoparticle stability from this. Both “stability maps” for particles across Europe look very much similar.

Similar to the above is the question if tests should be performed in synthetic test solutions or in filtered natural surface waters. The latter is attributed to more realism towards the situation in the environment. However, the composition of surface waters is extremely complex and can only with difficulties be reproduced in the laboratory. Hence and accepting the fact of local and temporal variability of natural surface water composition, the results from such a testing cannot be compared to other tests using different surface water, nor interpreted because the reason for the observed reaction will not be clear. Apart from sulfate the constituents important for nanoparticle dispersion stability are represented in the test and can be reproduced everywhere with high accuracy. We therefore conclude that using real waters would lead to scattered and more contradicting results without giving access to the reason of this compared to the synthetic solutions used in the proposed routine.

Also, the use of a certain type of NOM might bring some problems. The suggested purified SRNOM has only limited availability and is expensive. It has been already shown above that the purification and absence of elevated concentrations of impurities in the NOM is of critical importance for the outcome of the test routine. Because it leads to increased instability of the tested ENPs and autoagglomeration of the DOM, especially at lower pH. However other pure or later purified NOM materials may be used as alternatives. The impact on the dispersion stability is to a certain extent depending on the type, nature, origin and purity of the DOM, but applied at sufficiently high concentrations, as proposed in the test routine, these differences will be of less importance for the outcome of the test.

Currently the test system provides only qualitative information if a dispersion is relatively stable or not. For modeling purposes information like agglomeration rate or similar would be preferable. To be able to be included into later nanoparticle fate models a way to generate values from the dispersion stability testing which fit to the requirements of the models are required. The linking of stability tests and model parameter input is currently explored in the EU project NanoFase.

The OECD TG developed based on the research presented in the report is intended for OECD council adoption and then will be available at the webpage on OECD Guidelines for the Testing of Chemicals: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

5 Review on nanomaterials transformations reactions

The proposed TG considers no transformation of particles during the test. We here summarize currently relevant transformations of NPs in natural waters and in related systems. These could be taken into account when conducting the tests.

When released to the environmental conditions, ENPs can undergo various surface transformations (Dumont, et al. 2015, Mitrano, et al. 2015) that influence their behavior, transport and toxicity in the environmental aquatic systems. Among most abundant natural transformations are reactions of sulfidation, redox reactions (Ma, et al. 2014), processes of dissolution (Choi, et al. 2008) and exchange of surface moieties (Kaegi, et al. 2013). These transformations have to be properly considered to predict and monitor the environmental impact of ENPs and their possible toxicity. This part provides a condensed review on the transformation reactions of ENPs in the environmental systems.

5.1 Sulfidation

Sulfidation is a process that occurs both in the environment and anthropogenic systems. It is seriously considered when discussing the reduction of nanoparticle toxicity, especially in regard to silver nanoparticles (AgENPs), since sulfidation is major transformation product for Ag ENPs (Devi, et al. 2015, Starnes, et al. 2015), but affects in principle all particles composed from chalcophil elements. In general, sulfidation is known to decrease the toxicity of released Ag ENPs that is proved by numerous studies. For example, Daniel Starnes and co-workers investigated the impact of sulfidation on the bioavailability and toxicity of silver nanoparticles to *Caenorhabditis elegans* (Starnes, et al. 2015). They found that Ag ENPs greatly influence the reproduction cycle of organisms. Sulfidation decreased the toxicity of Ag ENPs for all examined endpoints. Less accumulated Ag was found in the organisms in case sulfidation was applied. Choi et al. (Choi, et al. 2008) showed that even a stoichiometric amount of sulfidation can decrease Ag ENPs toxicity to nitrifying bacteria up to 80%. Recently, another important report by Levard et al. (Levard, et al. 2014) showed that even very low degrees of sulfidation significantly decrease the toxicity of Ag ENPs against four diverse organisms; *Danio rerio*, *Fundulus heteroclitus*, *Caenorhabditis elegans*, and the aquatic plant *Lemna minuta*, clearly demonstrating that sulfidation could definitely be a potential mechanism for mitigating nanoparticle toxicity. Finally, Devi et al. (Devi, et al. 2015) have studied the effects of Ag ENPs sulfidation on the toxicity of these nanoparticles in case of vertebrates. The study has proved on the example of zebrafish that sulfidation of Ag ENPs resulted in significant decrease in liver oxidation stress, alteration of detoxification enzymes and affection of brain acetylcholinesterase activity.

Sulfidation of nanoparticles in the urban/ anthropogenical areas has been also widely studied. Recently, Kim et al. (Kim, et al. 2010) identified and characterized silver sulfide (Ag_2S) nanoparticles in sewage sludge products using analytical high-resolution transmission electron microscopy. Levard et al. (Levard, et al. 2014) investigated sulfidation of PVP-coated Ag ENPs and found out that it resulted in decreased silver ion mobility and also decreased the dissolution rate. Doolette et al.(Doolette, et al. 2013) investigated transformation of PVP coated silver nanoparticles in a simulated wastewater treatment process and the effect on microbial community. The study showed that transformed silver nanoparticles did not affect nitrification of methanogenesis in the predicted wastewater silver concentrations. Thus Ag NPs were not expected to affect the functioning of wastewater treatment plants. However Ag NPS were found to be able to negatively affect sub-dominant wastewater microbial communities. Surface speciation of Ag was also investigated in sewage sludge/biosolids on the example of three different types of Ag ENPs, and AgCl ENPs at environmentally realistic concentrations (Lombi, et al. 2013). The results showed that surface functionality or different compositions of the ENPs did not prevent the formation of Ag_2S. Silver sulfides were stable over a six month period of the experiment. The fate and transformation of Ag ENPs in urban wastewater systems was studied by Kaegi et al (Kaegi, et al. 2013). Authors report that sulfidation process of Ag ENPs is strongly dependent on their size. Starnes et al. investigated dissolution, uptake and toxicity of Ag nanoparticles before and after their sulfidation towards a model soil organism *Caenorhabditis elegans*. It was reported that reproduction was the most sensitive feature influenced by both tested materials. Sulfidation was shown to decrease the solubility of Ag nanoparticles and to reduce the bioavailability of intact Ag nanoparticles.

Silver nanoparticles are widely known for their use in textiles. It has been previously shown that significant amount of Ag ENPs are released upon washing. The solid phase speciation of Ag in original and washed silver textiles was studied by Lombi et al (Lombi, et al. 2014). The original Ag speciation in the textiles was found to vary between different materials with Ag(0), AgCl, Ag_2S, Ag–phosphate, ionic Ag etc. Washing with two different detergents resulted in significant changes in Ag-speciation. For some textiles the two detergents induced similar transformation, in other textiles they resulted in very different Ag species. Various Ag species were found to coexist before and after washing. Another study of nanoparticles released from the textiles during the wash was performed by D. Mitrano et al (Mitrano, et al. 2014). Authors use laboratory washing machine to simulate the typical washing process of a household. Authors reported that textiles treated by “conventional” and “nano” silver may be a source of silver nanoparticles in the washing solution. What’s more, it was found that textiles treated with the “conventional” silver have equal or greater propensity to form nano-silver particles than those textiles treated with nano-silver. A wide overview study on environmental transformations of silver nanoparticles and its impact on stability and toxicity was performed by Levard et al. (Levard, et al. 2012). Sulfidation is shown as one of the most abundant particle transformation reactions. Authors discuss some unanswered questions about Ag ENPs sulfidation, such as sulfidation grade, surface structure, composition and morphologies. Effect of ozone treatment on nano-sized silver sulfide in wastewater effluent was studied by Thalmann et al. (Thalmann, et al. 2015) The study is of a particular interest, since ozonation is used for eliminating organic micropollutants from wastewaters. The study has shown that ozonation results in the oxidation of silver sulfide and, consequently, in the increase of the Ag toxicity in the effluent.

As for sulfidation of other ENPs, numerous studies are devoted to CuO and ZnO ENPs. For example, Ma et al. (Ma, et al. 2014) investigated sulfidation of CuO ENPs in dependence to various S/Cu molar ratios. X-ray absorption spectroscopy, X-ray diffraction, and transmission electron microscopy indicate the coexistence of numerous copper sulfide (Cu_xS_y) species including crystalline CuS (covellite), and amorphous (Cu_xS_y) species at ambient temperature. Similar study was made by, Ma et al. (Ma, et al. 2013). The extent of sulfidation was found to depend on sulfide concentration. About 100% conversion was obtained in 5 days when sufficient amount of sulfide was present. It was also shown that sulfidation of ZnO NPs occurs by a dissolution and re-precipitation mechanism. Sulfidation also led to NP agglomeration and a decrease of surface charge.

5.2 Reduction-Oxidation transformation reactions of nanoparticles

Reduction and oxidation are coupled processes that involve transfer of electrons between chemical substances. Many ENPs are based on materials that undergo such reactions. Among them are silver (Henglein 1998, Lok, et al. 2007), iron (Liu, et al. 2005) and cerium (Baalousha, et al. 2010). Several studies dealing with various effects of RedOx reactions were observed. Parnklang (Parnklang, et al. 2015) has shown, that rapid transformation of spherical Ag ENP to nanoplates can pass in 2 minutes just through addition of H_2O_2, while presence of Polyvinylpyrrolidone (PVP) delayed the transformations. Thus the selective fabrication of nanoparticles was possible by variation of H_2O_2 and PVP amounts in the reaction mixture. Structural transformation of CeO_2 ENPs was observed by Wang et al. (Wang, et al. 2009). By means of transmission electron microscope they observed the atomic level of redox processes in individual CeO_2 ENPs aiming to clarify mechanism of their catalytic activity. Relations between redox state of Iron-Based nanoparticles and their toxicity toward *Escherichia coli* was tracked by Auffan et al (Auffan, et al. 2008). It was found that chemically stable nanoparticles (γ-Fe_2O_3) have no apparent cytotoxicity while nanoparticles containing ferrous and, particularly, zerovalent iron are cytotoxic. The cytotoxic effects were mainly related with an oxidative stress. Lok et al. (Lok, et al. 2007) studied the antibacterial properties of partially oxidized Ag ENPs. The paper states that partially (surface) oxidized Ag ENPs have antibacterial activities, but zero-valent nanoparticles do not. An overview study on light-induced redox reactions in nanocrystalline systems was made by A.Hagfeldt and M. Graetzel (Hagfeldt, et al. 1995). They focused on the factors that govern electron transfer reactions in colloidal semiconductor assemblies composed of TiO_2, ZnO, CdSe, CdS, WO_3, Fe_2O_3, SnO_2 and other particles. For example, the rate of oxygen reduction was found to be one of the important factors increasing the efficiency of TiO_2 photocatalysts. Thus surface modification of TiO_2 by chelating groups causing oxygen reduction would also cause an increase in the efficiency of photocatalist. Navrotsky et al. (Navrotsky, et al. 2010) dedicated their paper to the thermodynamically driven shifts in redox equilibria of transition metal oxides NPs. They investigated cobalt, iron, manganese and nickel oxide systems and showed how the surface energy influences their redox equilibria and phase stability. Spinels (M_3O_4) were found to have lower surface energies than metals (M), rocksalt oxides (MO), and trivalent oxides (M_2O_3) of the same metal.

5.3 Transformations and removal of surface organic/ inorganic coatings

One of the primary need of various organic or inorganic coatings is the stabilization of particle dispersion through creating the electrostatic, electro-steric or purely steric repulsion between particles. Among the used and most abundant organic coatings are citric acid, proteins (Sanghi, et al. 2009), various polymers and polysaccharides (Dahl, et al. 2007, Gigault, et al. 2013). As inorganic coating borate, chloride and carbonate are used while coating with SiO_2 and Al_2O_3 may lead to core-shell particles depending on the thickness of the coating (Piccapietra, et al. 2012). The fate of the coated particles is in a large extent determined by properties of the coating but also depends on the media. Thus the stability of Ag ENPs particles was tested in various media by e.g. Liu et al. (Liu, et al. 2012a), Römer et al. (Römer, et al. 2011), Zook et al. (Zook, et al. 2011). Tejamaya et al. (Tejamaya, et al. 2012) investigated stability of organic coated Ag ENPs over time in OECD media (pH=6.9) and 10 times diluted OECD media (pH=7.3). As well Cl^- ion in the OECD media was replaced by either nitrate (pH=6.9 /7.4) or sulfate ions (pH=7/ 7.2) for 1 and 1/10 concentrations of media respectively. The study revealed the stability of PVP-coated particles over 21 days, while those coated with citrate and PEG were unstable already after 3 days. MacCuspie (MacCuspie 2011) investigated the pH influence on the stability of organic coated AgNPs. He found that stability of Ag citrate coated ENPs can be related with the speciation of citric acid. Stability of BSA coated particles appears mostly due to steric effect provided by BSA molecules. Starch coated Ag ENPs showed greater stability than those Citrate and BSA stabilized, except at pH=10. Kittler et al. (Kittler, et al. 2010) have studied the dissolution of citrate and polyvinilpyrrolidone (PVP)-stabilized silver nanoparticles with different surface functionalization in ultrapure water at three different temperatures. In all cases only partial dissolution of particles has been observed, up to 90% of particle weight. Rate and degree of dissolution was related to surface functionalization and temperature. The resulted “aged” silver nanoparticles were found to be much more toxic to stem cells than freshly prepared ones due to increased release of Ag^+ ions. Virkutyte et al. (Virkutyte, et al. 2012) studied the removal of protection aluminum hydroxide coating in TiO_2-based sunscreens by ingredients of swimming pool water. It was found that chlorine in the swimming pool water was mainly responsible for disintegration of $Al(OH)_3$ protective layer of the TiO_2 particles. The highest redistribution of Al was achieved at Cl concentrations from 3.5 to 7 ppm. Auffan et al. (Auffan, et al. 2010) dealt with the similar problem, investigating structural degradation at the surface of a TiO_2 based nanomaterial used in the cosmetics. This material is composed of TiO_2 core, coated with two protected layers of $Al(OH)_3$ and polymethylsiloxane. It was discovered that upon contact with water at pH=5 and low ionic strength the investigated material becomes hydrophilic and aggregates. During this process about 90% of Si from organic coating was desorbed and polymethylsiloxane that remained at the surface was oxidized. At the same time $Al(OH)_3$ coating was also affected but stayed adsorbed at the particle surface and prevented the formation of superoxide ions from the photoactive processes that could happen on the TiO_2 core.

5.4 Interactions with NOM

NOM coatings on ENPs can significantly affect such processes as agglomeration and sedimentation, homo- and heteroagglomeration (Luo, et al.2016). Studies comparing the effects of NOM on the homoagglomeration of ENPs with different surface chemistries/ surface coatings have shown that NOM provides additional stability to the dispersions of nanoparticles. Thus NOM was proved to stabilize Au ENPs coated with citrate, tannate, etc. (Stankus, et al. 2011). Delay et al. (Delay, et al. 2011) investigated the interactions and stability of Ag ENPs in aqueous phase in order to study the effect of different NOM concentrations on the Ag ENP dispersion stability. They concluded that stabilization occurs due to the adsorption of NOM molecules onto the surface of Ag ENPs and has a steric nature. This is not expected to be the general case and contrasted by Zhang et al. (Zhang, et al. 2009) who found that NOM significantly increased the stability of ZnO, NiO, TiO_2, Fe_2O_3 and SiO_2 ENPs in aqueous media with a relatively small electrolyte concentration, due to development of negative surface charge, hence pointing towards an electrostatic effect. Quik et al. (Quik, et al. 2010) studied the influences of NOM presence on the dispersion stability of CeO_2 ENPs in deionized water and suspensions of algae growth medium. It was found that in presence of NOM, up to 88% of the initially added CeO_2 nanoparticles remained suspended in deionized water and 41% in algae growth medium after 12 d of settling. Gao et al. (Gao, et al. 2012) studied the influence of Suwannee River Humic Acid, (SRHA) on the dispersion properties and toxicity of Ag ENPs. It was reported that total silver content decreased with the concentrations of NOM greater than 10 mg Total organic carbon/L, meaning an increase in nanoparticle agglomeration and settling. However it was shown that NOM did not significantly influence the equilibrium of ionic silver present in the solution. Exposure of Daphnia to the dispersion of investigated silver particles revealed a linear decrease in their toxicity with increasing NOM. Interaction with NOM has been also used to reduce bacterial toxicity for Ag(0) (Fabrega, et al. 2009) and Fe(0) (Li, et al. 2010). In all cases, NOM was shown to screen the original properties of ENPs surfaces, either by direct coating or by minimizing dissolution. NOM was also found to alter CeO_2 NM oxidation state (Baalousha, et al. 2010) and to reduce Ag^+ to Ag NPs (Hou, et al. 2013). It was concluded, that such changes in the redox state of the ENPs can readily affect their toxicity. Thio et al. (Thio, et al. 2011) investigated the role of NOM on the agglomeration and deposition of TiO_2 ENPs onto silica surfaces, similar to those found in natural soils and sediments. They found that NOM drastically increased the stability of TiO_2 nanoparticles under most conditions, due to the combined electrostatic and steric effect. Presence of NOM significantly hindered TiO_2 deposition onto silica surfaces even under high ionic strength. It was also found that NOM destabilized Au ENPs coated with citrate but stabilized those coated with mercaptoundecanoic acid in the presence of Ca^{2+}. Liu et al. (Liu, et al. 2012b) investigated the agglomeration behavior of citrate- (C-AuNP) and 11-mercaptoundecanoic acid functionalized gold nanoparticles (M-AuNP) to understand the role of coating in the agglomeration process. Electrolyte (NaCl) and NOM concentration and pH was chosen as the controlling hydro chemical factors. It was found that C-AuNP were stable up to 100 mM NaCl and M-AuNP up to 200 mM NaCl, far above the range normally encountered in freshwaters. Both particle types were agglomerating in waters with low pH when the surface functional group carrying the electrostatically stabilizing negative charge was protonated. Interestingly NOM did not improve the stability of M-AuNPs while acting as expected with C-AuNPs. The reason was seen in the fact that NOM was unable to adsorb to the M-AuNPs surface because of the covalently bound 11-mercaptoundecanoic acid, hence no stabilization was achieved, contrary to the only unspecific bound citrate, which could be replaced by the NOM. In a later work (Liu, et al. 2013) the same authors reported that under the influence of divalent cations (Ca^{2+} and Mg^{2+}) the NOM addition to M-AuNPs stabilized the particles in the presence of Ca^{2+} but not in the presence of Mg^{2+}, showing for the first time that the complexation of divalent cations in solution by NOM may also have a stabilizing effect by reducing the activity of the ions. Ca^{2+} is much more strongly bound by NOM than Mg^{2+}

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