DOSSIER ON TITANIUM DIOXIDE
- PART 3 - NM 101

Series on the Safety of Manufactured Nanomaterials
No. 54

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DOSSIER ON TITANIUM DIOXIDE
- PART 3 - NM 101
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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

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PREAMBLE

In November 2007, OECD’s Working Party on Manufactured Nanomaterials (WPMN) launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The outcomes of the Testing Programme were intended to assess the applicability of the existing test guidelines to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions. Understanding the properties of MNs is crucial to choose appropriate strategies for hazard identification, risk assessment or risk management measures. The Testing Programme involved delegations from OECD member countries, some non-member economies and other stakeholders. The broad international representation, from a range of delegations enabled the programme to pool expertise and resources without which this programme would not have been possible.

Before launching the Testing Programme, the WPMN first identified a broad list of possible nanomaterials, and the list was later adjusted to a final selection of eleven MNs for testing. This list comprised: i) fullerenes (C60); ii) single-walled carbon nanotubes (SWCNTs); iii) multi-walled carbon nanotubes (MWCNTs); iv) silver nanoparticles; v) titanium dioxide; vi) cerium oxide; vii) zinc oxide; viii) silicon dioxide; ix) dendrimers; x) nanoclays; and xi) gold nanoparticles. One fundamental criterion for selecting these materials was that they should be either in commercial use at the time or expected to be in the near future. At the same time, other considerations were also given attention, such as the production volume of the materials, the likely availability of such materials for testing and the existing information that would readily be available on the materials.

It was also agreed that 59 endpoints would be addressed for each material corresponding to the following categories: i) nanomaterial information/ identification; ii) physical-chemical properties and material characterisation; iii) environmental fate; iv) toxicological and eco-toxicological effects; v) environmental toxicology; vi) mammalian toxicology; and vii) material safety. These endpoints were judged to be most important based largely on the general experience of testing chemicals, while taking into account the potentially different or new properties of nanomaterials. It is worth noticing that it was not expected that testing for all of the listed endpoints would be necessary for each of the selected MNs.

To assist with the Testing Programme, the WPMN developed two documents: i) a Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials [ENV/JM/MONO(2009)21]; and ii) Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme (Guidance Manual) in 2009, which was subsequently updated in 2010

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1 The OECD Test Guidelines are a collection of internationally agreed test methods used by government, industry and independent laboratories. They are used to determine the safety of chemicals.

http://www.oecd.org/chemicalsafety/testing/oecgdraftguidelinesfortesttestingofchemicals.htm

2 Originally Iron nanoparticles, Aluminium, Carbon black, and Polystyrene were suggested but later withdrawn and replaced by gold nanoparticles.

3 As specified in the Guidance Manual, “address” includes the term “completed” which provides that all dossiers will contain the identified endpoint information. Note that for some endpoints (for example, solubility) it is specified that the endpoint must be “completed”. In such instances “completed” means that all Dossiers will be providing this endpoint information.
The objective of this Guidance Manual was to guide sponsors\(^5\) in the testing of the materials while ensuring that the information collected was reliable, accurate, consistent and therefore also comparable. The Guidance Manual addressed a whole range of issues including the organisation of the work.

The *Guidance Manual* contains detailed information on the selected endpoints for testing and recommendations on sample preparation and dosimetry.

The *Guidance Manual* also described the development of *Dossier Development Plans* (DDPs). These plans were prepared by Lead sponsors, Co-sponsors together with contributors to describe the specific plan for the testing of each nanomaterial including when and where the testing will be undertaken and by whom. The DDPs also included information on the materials to be tested as well as information on issues such as sample preparation and dosimetry. Each of the DDPs was prepared and reviewed by the WPMN before testing work began.

Based on the lessons learned during the Testing Programme, the WPMN also developed *Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* [ENV/JM/MONO(2012)40]. This latter document is an update of an earlier text first published in 2010.

The work on OECD’s Testing Programme was completed by the end of 2013. In June 2014 the WPMN agreed that for each nanomaterial the dataset would be published in IUCLID printed format\(^6\)?. The document will include the protocols and methods to allow their wider use (regulators and researchers).

The dataset in this document has been declassified and made publicly available and it is expected regulators and researchers will wish to use it. Due to a broad dissemination of the data and the exploratory setting in which they were developed there are a number of limitations in using the data of which potential users should be aware. The programme focused on answering scientific questions in the field of the OECD test guidelines but not to provide conclusions on the hazard or risk of the materials selected. The data contained within these dossiers is raw data and has not been evaluated by either the programme sponsors or the WPMN. Any conclusions found within these dossiers are under the responsibility of the researchers who made them. The absence of data for some endpoints may be a gap for some endpoints but for other endpoints there may not if the data was not considered necessary. Although the programme ensured a broad participation of many stakeholders it was not intended to arrive at any pre-defined regulatory datasets requirements or risk assessment decisions. It was recognised from the beginning that the exploratory nature of the work would require subsequent follow-up work for example to review the

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\(^4\) It is worth noting that while the *Guidance Manual for Sponsors* was primarily intended as a guide to WPMN’s Testing Programme, it is also expected that it will be of value to anyone involved in testing NMs.

\(^5\) The Guidance Manual noted, for example, that there could be three levels of participation to the programme. Lead sponsors, who would assume responsibility for conducting or coordinating all of the testing, determined to be appropriate for each of the endpoints for a specific nanomaterial. In some cases, “joint lead” arrangements were developed. Co-sponsors conducted some of the testing determined to be appropriate and feasible to address the endpoints for a specific listed nanomaterial. Contributors provided test data, reference or testing materials or other relevant information to the lead and co-sponsors.

\(^6\) IUCLID is a software programme for the administration of data on chemical substances. Although it was originally developed to fulfill requirements in the EU for the evaluation and control of the risks of existing chemical substances, it is used by many others.

\(^7\) SIAR = SIDS Initial Assessment Report (SIDS = Screening Information Data Set)
specific needs that may arise when performing risk assessment of nanomaterials. In this context, the programme's ultimate goal, to add to the knowledge of the properties of nanomaterials, would form a cornerstone.
As part of its Programme on the Safety of Manufactured Nanomaterials, OECD launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The Testing Programme mainly aimed to assess the applicability of the existing test guidelines to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions.

This document presents the Dossier of the Titanium Dioxide (TiO$_2$) manufactured nanomaterials which was prepared under the leadership of France and Germany. TiO$_2$ has been tested for a number of endpoints for: i) Nanomaterials Information / Identification; ii) Physical-Chemical Properties; iii) Environmental Fate; iv) Environmental Toxicology; iv) Mammalian Toxicology; and vi) Material Safety. The data is presented in an IUCLID$^8$ style format and includes the protocols and methods used (see Preamble). They are resulting from scientific literature and testing following harmonised guideline or protocols (like OECD Guidelines for the Testing of Chemicals)$^9$, or not.

France and Germany led the Testing Programme on nano-TiO$_2$. This included the determination of data from the tests already completed using nano-TiO$_2$, a number of new tests from dedicated research project, as well as coordinating inputs provided and tests performed by other participating countries and stakeholder from Austria, Canada, Denmark, Spain, Japan, Korea, United Kingdom, United States, European Union, and the Business and Industry Advisory Committee to the OECD (BIAC).

Aeroxide® P 25 (P25) was chosen as principle material meaning that all the relevant endpoints have been addressed for this material.

- **Aeroxide® P 25**
  - provided and delivered by Degussa/Evonik, Lot-Nr.: 4168112198
  - provided and delivered by EC/JRC, Lot-Nr.: 4168031098 (called NM105)
  - US-NIST used the certified material SRM 1898, which was synthesised by NIST with the same properties than P25

At the same time, it was recognised that the nano-TiO$_2$ placed on the market presents high variability in its composition. With this in mind, additional materials were selected for performing a selected number of endpoints that could allow some comparability. As a consequence this allowed testing a broad range of material’s characteristics and covering a broader range of exposure scenarios to human and the environment. These materials were:

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$^8$ IUCLID is a software program for the administration of data on chemical substances. It was originally developed to fulfil requirements in the EU for the evaluation and control of the risks of existing chemical substances. It is specifically relevant in the context of an international programme for the initial assessment of chemical substances.

$^9$ http://www.oecd.org/env/testguidelines
The materials were delivered to the participating laboratories including: i) product information; ii) certification of analysis; iii) storage conditions; and iv) Safety Data Sheet.

Material provided by EC/Joint Research Centre was bought from the commercially available sources or provided by the manufacturer. To assure the traceability, the materials delivered by the EC/JRC were homogenised, sub-sampled and kept under inert atmosphere according to paragraph 42 of the Guidance Manual for Sponsors before the delivery to the participating laboratories.

Finally, a literature review on TiO$_2$ was performed to gather all the available information on the selected nanomaterials, even though it was not necessarily from the same batches.

Due to the large amount of information generated throughout the OECD Testing Programme on TiO$_2$, the Dossier has been split in 6 parts, as follows:

- **Part 1: NM 105** (P25)
- **Part 2: NM 100** (Tiona AT-1 (non-nano reference))
- **Part 3: NM 101** (Hombikat UV 100)
- **Part 4: NM 102** (PC105)
- **Part 5: NM 103** (UV TITAN M262 (Sachtleben))
- **Part 6: NM 104** (UV TITAN M212)

Each part includes Annexes.

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10 Cristal Global handed over its material to EC/JRC at a later stage of the test programme.
11 Cristal Global handed over its material to EC/JRC at a later stage of the test programme.
In the following document, an overview of the testing results, within the TiO$_2$ OECD Testing Programme, are presented. Detailed information on results and tests performed can be found in the technical dossiers of the particular TiO$_2$ nanomaterials.

During the elaboration of the dossier and because of variation observed for the same test performed with the same NM for one specific endpoint, it becomes an evidence that for an hazard assessment a well-considered review of the data for each end point has to be performed including the appropriateness of the test performances, information on exposure as well as information on the state of the NM within the test. Consequently, the lack of information about the state of the nanomaterial during the test performance (e.g. degree of agglomeration, interaction with other substances, different media used) conducts to a realistic exposition unknown.

Data within the dossier was gained by review of the literature as well as national and international projects, in particular like the European joint action Nanogenotox$^{12}$, which has covered both some mammalian toxicology and physical-chemical characterisation endpoints of the dossier or projects of the environmental research plan of the German Federal Ministry of Environment, Nature Conservation, Building and Nuclear Safety.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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$^{12}$ Nanogenotox is a European joint action, managed by France with 11 participating European countries and 17 laboratories. The list of participants is in the annex. (www.nanogenotox.eu)
ACKNOWLEDGMENTS

The OECD Secretariat and the Working Party on Manufactured Nanomaterials is which to thank France and Germany for leading the Testing Programme for Titanium Dioxide. In particular, Nathalie Thieriet from the Agency for Food, Environmental and Occupational Health & Safety “ANSES” (France), and Kathrin Schwirn from the Federal Environmental Agency UBA (Germany) who coordinated and led the testing of Titanium Dioxide, and Frank Herzberg from German Federal Institute for Risk Assessment (BfR) who reviewed the literature. We are also truly grateful to those delegations that had participated in the testing:

**Austria:**
- Vienna University, Department of Environmental Geosciences
- Graz University, Institute of Pharmaceutical Science

**Canada:**
- McGill University, Department of Chemical Engineering
- NRC-BRI, Applied Ecotoxicology Group
- Trent University, Environmental & Resource Studies Dept.
- Health Canada, Environmental Health Science and Research Bureau
- Health Canada, Healthy Environments & Consumer Safety Branch
- Wilfrid Laurier University, Institute for Water Science
- University of Victoria, Department of Biochemistry and Microbiology
- HydroQual Laboratories
- University of Alberta, Biological Sciences

**Denmark:**
- Technical University of Denmark, Department of Environmental Engineering

**France:**
- Nanogenotox Partner's
- ANSES, French Agency for Food, Environmental and Occupational Health & Safety (France), The Toxicology of Contaminants Unit, The Environmental Inorganic Contaminants and Mineral , The Department of Information, Communication and Dialogue with Society , The European and International Affairs Unit
- BfR, Federal Institute of Risk Assessment (Germany), The molecular toxicology unit of the Department of Safety of Consumer Products
- CEA, French Atomic Energy Commission (France), The Materials Sciences Division, The Life Sciences Division
- CODA-CERVA, Veterinary and Agrochemical Research Centre (Belgium), The Electron Microscopy unit
- EC/JRC, Joint Research Centre, Institute for Health and Consumer Protection (IHCP)
- Nanotechnology
FIOH, Finnish Institute of Occupational Health (Finland), The New Technologies and Risks laboratory
IMB BAS, Roumen Tsanev Institute of Molecular Biology Bulgarian Academy of Sciences (Bulgaria), The Medical and Biological Research Laboratory
IMC BAS, Institute of Mineralogy and Crystallography Bulgarian Academy of Sciences (Bulgaria), Central Laboratory of Mineralogy and Crystallography
INRS, The Medical and Biological Research Laboratory, Aerosol Metrology Laboratory and the Inorganic Analysis and Aerosol Characterization Laboratory, Carcinogenesis and Developmental Toxicology Laboratory, Pollutants and Health Department
INSA, National Health Institute Doutor Ricardo Jorge (Portugal), The Genetic Toxicology R&D Unit
IPH, Scientific Institute of Public Health (Belgium), The laboratory of toxicology
IPL, Insitut Pasteur of Lille (France), The Genetic Toxicology Laboratory
ISS, Istituto Superior di Sanita (Italy), The Food and Veterinary Toxicology Unit
LNE, Laboratoire National de metrolgie et d'Essais, Laboratoire National de metrolgie et d'Essais
NIOM, The Nofer Institute of Occupational Medicine (Poland), The Laboratory of Molecular Toxicology
NRCWE, National Research Centre for the Working Environment (Denmark), Nanotoxicology and Occupational Hygiene Group
RIVM, National Institute for Public Health and Environment (The Netherlands), The Laboratory for Health Protection Research
UAB, Universitat Autonoma de Barcelona (Spain), The Group of Mutagenesis

Germany:
Institute of Energy and Environmental Technology (IUTA ), Air Quality & Sustainable Nanotechnology
Fraunhofer Institute of Toxicology & Experimental Medicine, Inhalation Toxicology & Chemical Risk Assessment
Fraunhofer Institute for Molecular Biology and Applied Ecology
RWTH Aachen, Institute of Ecochemistry, Ecology, and Ecotoxicology
University Frankfurt Main, Institute for Ecology, Evolution and Diversity
Technical University Dresden, Institute of process engineering and environmental technology
Hamburg University of Applied Sciences
Federal Institute for Materials Research and Testing, Materials and Air Pollutants

Japan:
National Metrology Institute of Japan, Advanced Industrial Science and Technology (AIST)

Korea:
Dongduk Women's University, College of Pharmacy
Hanyang University, Laboratory of Nanoscale Characterisation & Environmental Chemistry
Korea Research Institute of Standards and Science, Korea Research Institute of Standards and Science Division of Industrial Metrology
Seoul National University, School of Chemical & Biological Engineering
Kyung Hee University, Department of Applied Chemistry
Korea University, School of Life Science & Biotechnology

**Spain:**
INIA, Departamento de Medio Ambiente

**USA:**
NIST, Nanoparticle Measurements & Standards
EPA, National Health and Environmental Effects Research
EPA, Ecology Division
FDA, National Center for Toxicological Research

Finally, we would also like to acknowledge the effort done by the EC/Joint Research Centre in providing the materials, homogenised, sub-sampled and kept them under inert atmosphere before they delivered them to participating laboratories; as well as TDMA in providing some of the materials to JRC.
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Substance: Titanium Dioxide (Hombikt UV Titan; NM 101)

1. GENERAL INFORMATION

1.1 Identification

Substance identification
Chemical name  Titanium Dioxide (Hombikt UV Titan; NM 101)

Type of substance
Composition  other: Existing Chemical
Origin  element

1.2 Composition

Degree of purity

Constituents

| Reference substance | ti  
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1.3 Identifiers

1.4 Analytical information

1.5 Joint submission

1.6 Sponsors

1.7 Suppliers

1.8 Recipients

1.9 Product and process oriented research and development

2. CLASSIFICATION AND LABELLING

3. MANUFACTURE, USE AND EXPOSURE

3.1 Technological process

3.2 Estimated quantities

3.3 Form in the supply chain

3.4 Identified uses and exposure scenarios

3.5 Uses advised against

3.6 Waste from production and use

3.7 Exposure estimates

3.8 Biocidal information

3.9 Application for authorisation of uses
4. PHYSICAL AND CHEMICAL PROPERTIES

4.1 Appearance

4.2 Melting point/freezing point

4.3 Boiling point

4.4 Density

4.5 Particle size, size distribution

*Endpoint study record: Particle size, size distribution by INIA*

**Administrative Data**

- **Purpose flag**: ( ) robust study summary ( ) used for classification ( ) used for MSDS
- **Study result type**: experimental result

**Data source**

**Data access**

other: performed and provided by INIA, Spain

**Materials and methods**

**Methods**

TEM

**Results and discussions**

**Mean diameter**

< 10 nm
Remarks on results including tables and figures
The representative TEM pictures confirm the particle size provided by the supplier (8-10nm).

Department de Medio Ambiente (Department of Environment)
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Madrid, Spain

Date: 21.06.12
TEM NM 101 Hombikat .doc
Figure 3: NM-101

Overall remarks, attachments
Attached background material

Endpoint study record: TEM_ by University of Graz

Administrative Data
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Author Karl Franzens University Graz, Institute of Pharmaceutical Sciences, Pharmaceutical Technology, Dr. Eva Roblegg and Sandra Blass
Title: Sponsorship Program: Titanium Dioxide Report

Data source
Data access
other: performed and provided by University of Graz

Materials and methods
Methods
TEM
Overall remarks, attachments
**Endpoint study record: Particle size, size distribution by TEM by NANOGENOTOX**

**Administrative Data**

- **Purpose flag**: key study (X) robust study summary ( ) used for classification ( ) used for MSDS
- **Study result type**: experimental result

**Data source**

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<tr>
<td><strong>Author</strong></td>
<td>Keld Alstrup Jensen</td>
</tr>
<tr>
<td><strong>Year</strong></td>
<td>2012</td>
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<tr>
<td><strong>Title</strong></td>
<td>D4.2: Transmission electron microscopic characterisation of NANOGENOTOX nanomaterials. Key intrinsic physicochemical characteristics of NANOGENOTOX nanomaterials</td>
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<td><strong>Bibliographic source</strong></td>
<td>NANOGENOTOX Deliverable no. 5 Final Report</td>
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<td><strong>Company study no.</strong></td>
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<td><strong>Report date</strong></td>
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**Data access**

other: Owner: NANOGENOTOX

**Materials and methods**

**Test guideline/method**

- **Qualifier**: equivalent or similar to
- **Guideline**: other guideline: NIST 960-1 Guideline
- **Deviations**: yes The general approach of the methodology is based on NIST 960-1 however it is not equivalent

**Methods**

TEM BF-TEM (Bright Field Transmission Electron Microscopy)

**Principles of method if other than guideline (including performance, material limits, other limits)**

The general approach of the methodology is based on NIST 960-1 however it is not equivalent.

**Details on methods and data evaluation**

1. To measure the characteristics of primary particles of a NM, the Feret Min and Feret Max were measured by CODA-CERVA following a systematic random sampling based on stereology at an appropriate magnification.
2. The Feret Max and Feret Min were measured and the Feret Mean was calculated as the mean of Feret Min and Feret Max. The aspect ratio was calculated as the ratio of Feret Max and Feret Min. [Feret diameter is the distance between two tangents on opposite sides of the particle, parallel to some fixed direction. Feret max is the maximum projected length and Feret Minimum the minimal one.] Micrographs were taken at 10 fixed positions determined by the microscope stage. On
these micrographs a grid with a mesh of 100 nm by 100 nm was placed at random. The primary particles on each tenth intersection, counted from left to right were measured. When no particle was located at this intersection, the horizontal grid lines were followed until a primary particle was located on an intersection. The ‘Detection module’ of iTEM was used for threshold-based detection of the NM. The contrast and brightness of the micrographs were optimized, the involved particles were enclosed in a pre-defined frame or region of interest and thresholds were set to separate particles from the background based on their electron density and size. Particles consisting of less than fifty pixels and particles on the border of the frame were omitted from analysis. For each particle, twenty-three quantitative parameters, (described in Table 1-attachment), are measured and considered relevant for its characterization. Each particle detected in a micrograph was identified by a unique number, written in the overlay of the image. This allowed the selection of data of individual particles and the postanalysis deletion of erroneously detected particles. Artefacts were characterized by their morphology and a grey value lower than the mean grey value of the background plus three times its standard deviation. Particles fulfilling this criterion were identified and deleted automatically and particles with an unusual morphology, judged to be artefacts based on visual inspection on the micrographs, were omitted manually from analysis. 

Descriptive statistics and histograms were calculated in Sigmaplot (Systat, Cosinus computing, Drunen, The Netherlands). The normality of the distributions of the measured parameters was tested with the Shapiro-Wilk and Kolmogorov-Smirnov tests, while the homogeneity of variances was tested with the Tukey test. The measured parameters were classified by principle component analysis using the SAS statistical software (SAS Institute Inc., Cary, NC, USA).

**Used Protocols**

1. Dispersion of the sample: NM sample was suspended in double distilled water at a concentration of 2.56 mg/ml and sonicated for 16 minutes using a Vibracell™ 75041 ultrasonifier (750 W, 20kHZ, Fisher Bioblock Scientific, Aalst, Belgium) equipped with a 13 mm horn (CV33) at 40% amplitude. This setup resulted in an average horn power of about 26 W and a sample specific energy of 2530 ± 20 MJ/m³. During sonication the samples were cooled in icy water with ice to prevent excessive heating. After sonication, the samples were diluted to a concentration of 0.512 mg/ml. Details of used procedure can be found in the nanogenotox dispersion protocol file.

2. Grid adjustment The charge of grid was adjusted in order to allow for the attachment of the negatively charged silica NM to the EM grid. Alcian blue pretreatment introduced positive charges on the surface of pioloform- and carbon-coated grids that tend to have a negative or neutral charge. (authors hand experience suggests that this approach is easier than the alternative based on glow discharging EM-grids with air to introduce negative charges and subsequent Mg²⁺ treatment, introducing positive charges). For TEM measurements the suspended NM was brought on pioloform- and carbon- coated, 400 mesh copper grids (Agar Scientific, Essex, England) that were pretreated with 1% Alcian blue (Fluka, Buchs, Switzerland). More details about the step by step procedures used for TEM analysis at Coda-Cerva can be found in protocols files.

**Used Protocols: attached files**

**Attached document**  D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1

**Remarks** Dispersion protocol

Remarks: Data in the report and details protocol in annex

Data gathering

Instruments
The samples were examined using a Tecnai Spirit microscope (FEI, Eindhoven, Netherlands) operation at 120Kvm at a spot size 3.

Calibration
Details for calibration in Semi-automatic and Automatic modes can be found in the protocol files. Basic Calibration: • For each NM three independent samples were analyzed. • Per sample, five micrographs were made with a 4*4 k Eagle CCD camera (FEI) at a magnification of 18500 times. • For the given microscope and camera configuration, this magnification corresponds with a pixel size of 0.60 nm and a field of view of 2.45 μm by 2.45 μm. (This implies a lower particle size detection limit of approximately 6 nm, supporting on the criterion of Merkus (HG. Merkus, Particle Size Measurements, 1Edn. Pijnacker: Springer 2009) that large systematic size deviations can be avoided if the particle area is at least hundred pixels.) • The field of view limits the upper size detection limit to 245 nm, one tenth of the image size as recommended in ISO 13322-1 (part 1, 2004)

Reproducibility

Test materials
Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier: Reference Material/Nanomaterial

Identity: NM-101

State of test material
other: fluffy powder

Results and discussions

Remarks on results including tables and figures
Figure 1 NM101: A) Representative TEM-micrograph showing the range in agglomerate and aggregate sizes. B) Selected TEM-micrograph showing that the sample contains two different aggregate types. One type consists of aggregates of 10-20 nm-size primary particles. The second type consists of coarser ca. 100 nm or larger, dense and rounded aggregates. B) Image taken at higher resolution showing that the aggregates consist of ca. 5 nm-size crystallites. Insert shows the electron diffraction pattern of anatase.

Table 1 Primary particle area equivalent circular diameter of the titanium dioxide NM analysed by different partners.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ECD (nm) ± SD (N); CODA-CERVA</th>
<th>ECD (nm) ± SD (N); INRS</th>
<th>Diameter (nm); IMC-BAS</th>
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<tr>
<td>NM-101</td>
<td>6*</td>
<td></td>
<td>4.5 ± 0.6**</td>
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Overall remarks, attachments
Attached full study report
D4.2_TEM_characterisation: ENV/JM/MONO(2015)17/ANN4

Applicant's summary and conclusion
Conclusions
very good correspondence between AFM and TEM values

Cross-reference to other study
4.6 Vapour pressure

4.7 N-octanol-water partition coefficient

4.8 Water solubility, hydrophilicity, dispersibility

4.9 Solubility in organic solvents / fat solubility

4.10 Surface tension

4.11 Flash point

4.12 Auto flammability

4.13 Flammability

4.14 Explosiveness

4.15 Oxidising properties

4.16 Oxidation reduction potential

4.17 Stability in organic solvents and identity of relevant degradation products

4.18 Storage stability and reactivity towards container material

4.19 Stability: thermal, sunlight, metals

4.20 pH

4.21 Dissociation constant

4.22 Viscosity

4.23 Additional physico-chemical information

Endpoint study record: composition by TGA by NANOGENOTOX

Administrative Data

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Data source

Reference

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<th>study report</th>
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<tr>
<td>Author</td>
<td>KA Jensen</td>
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<tr>
<td>Title</td>
<td>Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials</td>
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<td>NRCWE (DK)</td>
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<td>Report date</td>
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Data access

other: owner: NANOGENOTOX

Materials and methods

Endpoint investigated

other: mass lost by TGA

Details on methods and data evaluation

In a thermograviometric measurement a sample is heated in a gas (usually air, O2 or N2) and the weight of the sample is measured as a function of the temperature. The decomposition temperature and loss of mass may give information about the sample, e.g. water adsorbed to the surface of particles will evaporate around 100 °C, whereas most other associated or technically added organic coatings will evaporate or combust at higher temperature. A decomposition in several steps will indicate a non-homogeneous sample containing several different types of combustible compounds, which could in fact all be structurally different carbon nanotubes.

Instruments: For the thermogravimetric analysis (TGA) NRCWE used a Mettler Toledo TGA/SDTA 851e and an oxygen atmosphere. The heating rate was 10 K/min and the same temperature range from 25 °C to 1000 °C. The sample holders used for the TGA measurements were made of alumina and had a volume of 70 μL or 150 μL.

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier    Reference Material/Nanomaterial
Identity       NM-101

Any other information on materials and methods incl. tables

The SOP used for TGA analysis: Thermogravimetric Analysis (TGA) Renie Birkedal (NRCWE) based on NIST Recommended Practice Guide, Special Publication 960-19 General description of TGA is short for thermogravimetric analysis. The principle is measuring sample weight as a function of temperature in a given atmosphere at a given heating rate. TGA is measured according to information wanted and material investigated. If information about evaporation is wanted heating in N2 is recommended. If information...
about organic content is wanted heating in O2 or air is recommended, as this will insure combustion of all organic material. In order to make sure e.g. all organic material is decomposed, it is recommended to run to 1000 °C.

Materials and Chemicals:
- Powder (may be conditioned in a specific atmosphere and humidity conditions)
- Laboratory weigh (scale)
- Apparatus for thermogravimetric analysis

Procedure:
Sample preparation:
1. Weigh container.
2. Fill container with material. Do not stamp it, as this may affect the evaporation/decomposition temperature.
3. Weigh container and material.

For inorganic powder materials a minimum of 10 mg should be used – if possible more. These samples are usually quite homogeneous and this is usually a representative fraction of the sample. CNT samples are somewhat different. They are in many cases bundles, and these bundles may be different. At the same time these compounds often have a low density, and it is therefore difficult to measure a representative fraction in one or two measurements. The solution is many measurements and comparison of the data.

Selection of heating rate:
For inorganic materials only a minor fraction is expected to decompose, and a heating rate of 10°C/min is recommended. It is not assumed that there will be large weight losses for these materials, so this heating rate ensures a fast measurement and most likely still well defined weight losses. If the weight losses are not well defined a slower heating rate can be chosen. The NIST Recommended Practice Guide, Special Publication 960-19, Measurement Issues in Single Wall Carbon Nanotubes, recommends a heating rate of 5°C/min. This is chosen as a compromise between time and avoiding too much spontaneous combustion. For some carbon nanotubes 5°C/min is not slow enough to avoid spontaneous combustion. There is no spontaneous combustion with a heating rate of 2.5°C/min. The measurement time is very long, app 7 hours per measurement, but this is still recommended. In order to minimize measuring time it may be an option only to heat to 900°C or even lower.

Data treatment:
Compare TGA curve and curve for first derivative to find steps of weight loss. It is recommended to obtain several measurements to calculate the mean and standard deviation of the weight loss and the evaporation/decomposition temperatures. (the last is most easily found from the curve of the first derivative). The test of multiple samples also enables evaluation of sample homogeneity.

Results and discussions

Results
TGA measurements on the samples were performed once only as the quantities analyzed were sufficiently large to be representative, and the main purpose for these measurements has been to detect coating on the materials.

For NM101, there are two weight losses. The first and greatest loss occurs below 100°C, and is most likely due to adsorbed water. The second weight-loss event occurs around 200°C and is most likely due to an organic coating or associated organic matter.

![TGA of NM101](image)

![1st derivative of TGA measurement on NM101](image)

*Figure 0-2. Results from TGA measurement on NM101.*
Overall remarks, attachments

Attached full study report

Attached document D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks Dispersion protocol

Remarks Data in the report and details protocol in annex

Applicant's summary and conclusion

Conclusions

Significant mass loss up to ca. 100°C which is ascribed to trapped or adsorbed water (Figure 4-2). In this sample, a second episodic loss is observed at ca. 200°C, which is ascribed to associated organic matter or coating

Cross-reference to other study

Endpoint study record: composition by EDS by NANOGENOTOX

Administrative Data

Purpose flag key study (X) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source

Reference

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<th>Reference type</th>
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<td>Author</td>
<td>KA Jensen</td>
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<tr>
<td>Year</td>
<td>2013</td>
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<tr>
<td>Title</td>
<td>Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials</td>
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Data access

other: owner: NANOGENOTOX
Materials and methods

Endpoint investigated
other: composition by EDS

Details on methods and data evaluation

EDS is short for Energy-dispersive X-ray spectroscopy and may be available as an extra analytical tool in electron microscopes. The analysis is based on the fact that when hitting a material with charged particles, such as an electron beam, some of the electrons of the atoms in the matter under the beam will first be energized to higher orbital positions and then drop down to their appropriate energy level again during which X-rays are emitted. The emitted X-rays are characteristic for each element and have specific energetic wavelengths and energy patterns. Therefore an elemental composition can be quantified by analyzing the energy spectrum and intensities of the X-rays emitted during the analysis. EDS is mostly possible for Na and heavier elements. Lighter elements from Be and up may also be quantified depending on detectors and instrumental configuration. Oxygen is normally not analysed by SEM EDS, but may be calculated by difference or by converting all elements to oxides. When calculated by difference, as done in this work, the sum of all elements adds up to 100 wt%. Measurements may be made as semiquantitative or quantitative analyses using either standardless/internal instrument standard values or calibrated concentration-intensity curves using range of relevant metals, minerals and glass standards, respectively. In the present analysis, elements were reported as semi-quantitative results. Due to current quality of detectors and in-build standard references, such results are relatively reliable for major elements if the materials have sufficiently high thickness and low roughness. Samples were prepared by pelletizing a known amount of powder. The results are given in wt.% and parts per million (ppm) depending on the absolute concentrations in the sample materials.

Test materials

Reference Material/Nanomaterial and Sample identification number

Identifier | Reference Material/Nanomaterial
--- | ---
NM 101

Results and discussions

Results

<table>
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<th>Element</th>
<th>Concentration (ppm/wt%)</th>
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<td>Al</td>
<td>900</td>
</tr>
<tr>
<td>Si</td>
<td>2900</td>
</tr>
<tr>
<td>P</td>
<td>2700</td>
</tr>
<tr>
<td>S</td>
<td>2200</td>
</tr>
<tr>
<td>K</td>
<td>0</td>
</tr>
<tr>
<td>Ti</td>
<td>58.79</td>
</tr>
<tr>
<td>Cr</td>
<td>0</td>
</tr>
<tr>
<td>Fe</td>
<td>0</td>
</tr>
<tr>
<td>O</td>
<td>40.35</td>
</tr>
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Remarks on results including tables and figures

Table 0-2 Elemental concentrations by EDS measurements on TiO₂ performed at IMC-BAS.

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<tr>
<th>Sample</th>
<th>Al*</th>
<th>Si*</th>
<th>P*</th>
<th>S*</th>
<th>K*</th>
<th>Ti (wt%)</th>
<th>Cr*</th>
<th>Fe*</th>
<th>O wt%</th>
</tr>
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<tr>
<td>NM101</td>
<td>900</td>
<td>2900</td>
<td>2700</td>
<td>2200</td>
<td>58.79</td>
<td>0</td>
<td>0</td>
<td>40.35</td>
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Overall remarks, attachments

Attached full study report

Attached document | D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks | Dispersion protocol
Remarks
Data in the report and details protocol in annex

Applicant's summary and conclusion

Conclusions
Sample only contain minor elemental impurities. The presence of calc-alkali elements, S and Al support the analyses (XRD) with occasional observation of Na sulfate and boehmite.

Cross-reference to other study

Endpoint study record: composition by ICP_OES by NANOGENOTOX

Administrative Data

Purpose flag key study (X) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source

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Data access
other: Owner: NANOGENOTOX

Data protection claimed
yes, but willing to share

Materials and methods

Endpoint investigated
other: Elemental composition

Details on methods and data evaluation
All measurements were carried out with inductively coupled plasma-optical emission spectrometry(Varian 720-ES, Agilent Technologies), using the SemiQuant feature, which is designed to provide a fast estimate of the concentration of non-calibrated compounds in samples. The samples were screened for 68 elements (Figure 5-1) (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, P, Pb, Pd, Pr, Pt,
Rb, Re, Rh, Ru, Sb, Sc, Se, Si, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, W, Y, Yb, Zn, Zr). Sample preparation: To bring the NM sample in solution, 0.1 g was weighed in a 50 ml DigiPREP HT tube (SCP SCIENCE) and 2 ml of concentrated HF was added. The mixture was heated overnight at 80°C in a DigiPREP MS (SCP SCIENCE). After cooling, the volume was made up to 10 ml with doubledistilled water.

**Test materials**

**Test material equivalent to submission substance identity**

yes

**Reference Material/Nanomaterial and Sample identification number**

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<td>Identity</td>
<td>NM-101</td>
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**Results and discussions**

**Results**

*Table 0-3. Graphical summary table with the impurity ranges found in titanium dioxide.*

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Vial ID n°</th>
<th>Impurities</th>
<th>Impurities</th>
<th>Impurities</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Na, Mg, Al, Si, P, K, Ca, Cr, Mn, Fe, Co, Ni, Zr, Mo, Ag, Ba, La, W</td>
<td>&gt; 10 mg/g</td>
<td>&gt; 1 mg/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Al, Na (&gt;0.1%), P, S, Zr³</td>
<td>&gt; 1 mg/g</td>
<td>&gt; 100 µg/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K, Ca</td>
<td>&gt; 1 mg/g</td>
<td>&gt; 50 µg/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K, Zr³</td>
<td>&gt; 1 mg/g</td>
<td>&gt; 10 µg/g</td>
</tr>
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</table>

*Near 0.01%*

**Table 0-4. Overview of impurities detected in titanium dioxide NM.**

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Vial ID n°</th>
<th>Impurities 0.005 – 0.01%</th>
<th>Impurities 0.001 – 0.005%</th>
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</thead>
<tbody>
<tr>
<td>NM-101</td>
<td>1252</td>
<td>Al, Na (&gt;0.1%), P, S, Zr³</td>
<td>K, Ca</td>
</tr>
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<td></td>
<td>1265</td>
<td>Al, Na (&gt;0.1%), P, S</td>
<td>K, Zr³</td>
</tr>
</tbody>
</table>

**Overall remarks, attachments**

**Attached full study report**

**Attached document** D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1

**Remarks** Dispersion protocol


**Remarks** Data in the report and details protocol in annex
Applicant's summary and conclusion

Cross-reference to other study


4.24 Agglomeration/aggregation

Endpoint study record: Agglomeration/aggregation by INIA

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source

Data access
other: performed and provided by INIA, Spain

Materials and methods

Methods
DLS

Used Protocols

The Nanocare Protocol has been used for the preparation of aqueous suspensions of Titanium dioxide nanoparticles for six different concentrations and five different nanomaterials. Their aggregation/agglomeration has been measured by DLS (Zetasizer Nano Series, Malvern Instruments ZEN 3600), and their stability has been followed up for 14 days. Stocks suspension: Mass concentration: 1mg/L, 10 mg/L, 50 mg/L and 100mg/L. Solvent: Milli-Q water. The stock suspension was stirred at 900 rpm for 24 h at room temperature in a glass bottle wrapped with aluminium foil. Conditions: Glass Bottle (1L, borosilicate 3.3) Diluted suspensions: Mass concentration: 0.1mg/L and 0.01 mg/L. Nanocare protocol has been used for the preparation of media suspension of Titanium dioxide nanoparticles at also five different concentrations. Their aggregation/agglomeration has been measured by DLS, and their stability has been followed up for three days.

Data gathering

Test materials

State of test material
dispersion

Results and discussions

Particle size distribution in water showed, that agglomerates of the particles range between approximately 600 nm to 200 nm. The dispersions appear to be stable during the time of study. Particle size The measurements showed, that the size distribution in media is comparable to the size distribution in water (not measured for low concentration).

Remarks on results including tables and figures
Graphic 1: Particle size distribution in water at t =0h

1- Particle size distribution in media suspensions
Graphic 2: Particle size distribution at t = 0h

Graphic 3: Particle size distribution at t = 24h
Overall remarks, attachments
diagramm agglomeration.doc

Endpoint study record: Agglomeration/aggregation by University of Graz

Administrative Data
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source
Data access
other: performed and provided by Uni Graz

Materials and methods
Methods
other: PCS

Details on methods and data evaluation
As solid particles show low stability and a high tendency to aggregate in aqueous dispersions, our first goal was to produce a stable TiO\textsubscript{2} dispersion. Several pre-tests had been carried out with sample ID NM-105. These tests included coatings with sodium citrate and lecithin (as TiO\textsubscript{2} particles are known to be lecithin coated in sunscreen), as well as different sonication methods. Furthermore the effects of pH and ionic strength on the surface charge of the particles were investigated. The particles were characterized in
terms of their physico-chemical properties (i.e., i. size, ii. distribution, iii. agglomeration, iv. surface charge) with Photon Correlation Spectroscopy (PCS) using a ZetaSizer Nano-ZS (Malvern). Subsequently, all particles acquired from the OECD were characterized in terms of their physico-chemical properties using a Zetasizer NanoZS (Malvern) in different biological media. Furthermore, three different pre-treatment methods had been carried out (i no pre-treatment, ii probe sonification and iii ultrasound bath) in order to evaluate which method provides the best results. Data acquired is shown in the table below.

**Data gathering**

**Instruments**

ZetaSizer Nano-ZS (Malvern)

**Results and discussions**

These tests demonstrated that sonification with a probe sonifier leads to smaller particle sizes. However, the original particle size of 22 nm could not be achieved. Lecithin coated particles demonstrated 2-fold smaller diameters in MQ-water compared to uncoated particles. Sodium citrate coated particles showed smaller sizes in MQ-water, but agglomerated in PBS-buffer and cell culture medium. According to the zeta potential, uncoated (-33.5 mV) and sodium citrate coated (-39.3 mV) TiO2 particles dispersed in MQ-water exhibited high negative surface charges, which indicates a rather stable dispersion, whereas the zeta potential of lecithin coated particles was recorded at -3.79 mV in MQ-water, an indication for an unstable dispersion. Additional studies were performed to evaluate the influence of ionic strength and pH on the agglomeration behavior of NM-105. The results showed huge diameters and zeta potentials around 0, which implies that particles agglomerate in Na2HPO4-citric acid buffer and therefore, form an unstable dispersion.

**Remarks on results including tables and figures**

**Sponsorship Program: Titanium Dioxide Report**

Karl Franzens University Graz, Institute of Pharmaceutical Sciences, Pharmaceutical Technology, Dr. Eva Roblegg and Sandra Blass

Results of the particle characterization of NM101 in different biological media

<table>
<thead>
<tr>
<th>NM101</th>
<th>0.4 mg/ml TiO2 particles (NM101, 7nm, anatase, Fa. Hombikat) untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
<td><strong>Size (d.nm)</strong></td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>1609</td>
</tr>
<tr>
<td>PBS</td>
<td>1188*/5148</td>
</tr>
<tr>
<td>DMEM + L-Glutamine</td>
<td>1438*/5560</td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>1201*/5232</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>1278</td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>1406</td>
</tr>
</tbody>
</table>
### 0.4 mg/ml TiO2 Partikel (NM101, 7nm, anatase, Fa. Hombikat) 1 min Sonifier (40% Amplitude)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Size (d.nm)</th>
<th>Z-Average (d.nm)</th>
<th>PdI</th>
<th>Zeta Potential (mV)</th>
<th>monomodal</th>
<th>Zeta Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQ Wasser</td>
<td>719,5</td>
<td>500,9</td>
<td>0,274</td>
<td>-27,2</td>
<td></td>
<td>5,5</td>
</tr>
<tr>
<td>PBS</td>
<td>2254</td>
<td>1827</td>
<td>0,283</td>
<td>-19,7</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>DMEM + L-Glutamine</td>
<td>2854</td>
<td>2350</td>
<td>0,217</td>
<td>22,3*/-34,3/-92,0</td>
<td>-5,52</td>
<td>43,7</td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>678,5</td>
<td>521,2</td>
<td>0,232</td>
<td>-11,8</td>
<td></td>
<td>18,8</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>755,5</td>
<td>569,2</td>
<td>0,232</td>
<td>-15</td>
<td></td>
<td>19,9</td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>823,6</td>
<td>623,4</td>
<td>0,24</td>
<td>-13</td>
<td></td>
<td>16,9</td>
</tr>
</tbody>
</table>

### 0.4 mg/ml TiO2 Partikel (NM101, 7nm, anatase, Fa. Hombikat) 20 min US-bath

<table>
<thead>
<tr>
<th>Medium</th>
<th>Size (d.nm)</th>
<th>Z-Average (d.nm)</th>
<th>PdI</th>
<th>Zeta Potential (mV)</th>
<th>monomodal</th>
<th>Zeta Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQ Wasser</td>
<td>1111 */4077</td>
<td>1130</td>
<td>0,351</td>
<td>-27,5</td>
<td></td>
<td>3,67</td>
</tr>
<tr>
<td>PBS</td>
<td>1265*/4976</td>
<td>1276</td>
<td>0,238</td>
<td>-21,7</td>
<td></td>
<td>12,9</td>
</tr>
<tr>
<td>DMEM + L-Glutamine</td>
<td>1974*/4881</td>
<td>1992</td>
<td>0,247</td>
<td>3,6*/-42,5</td>
<td>-7,32</td>
<td>27,5</td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>1368*/420,5</td>
<td>668,7</td>
<td>0,282</td>
<td>-12</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>1073*/5046</td>
<td>1065</td>
<td>0,302</td>
<td>-11,3</td>
<td></td>
<td>13,6</td>
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<tr>
<td>DMEM + 10% FBS</td>
<td>1255*/5222</td>
<td>957,9</td>
<td>0,234</td>
<td>-11,5</td>
<td></td>
<td>14</td>
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</table>

### Results of the particle characterization with a Mastersizer 2000

<table>
<thead>
<tr>
<th>NM101</th>
<th>TiO2 Partikel (NM101, 7nm, anatase, Hombikat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Size(0.1) [nm]</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>16609</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>630</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>655</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>704</td>
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<tr>
<td>MQ Wasser</td>
<td>776</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>879</td>
</tr>
</tbody>
</table>
Overall remarks

The results show that in most cases the probe sonifier method leads to the smallest particle sizes, however, particles seemed to lose stability and some of them changed their positive surface charge to a negative surface charge. The ultrasound-bath method did not influence the particle size (compared to untreated particles), particles still stayed agglomerated, but these Agglomerates were stable in most cases. Furthermore, particle size distribution was evaluated with laser diffraction (LD) (Mastersizer 2000, Malvern). Laser diffraction is based on the fact that particles passing through a laser scatter light at an angle, which is directly related to their size. All particles had been suspended in MQ-water. During the measurements particles were stirred at 1500 rpm and sonified with 90% amplitude. The results demonstrate that all particles are highly aggregated and Ultrasonification for more than three minutes does not break up these aggregates.

4.25 Crystalline phase

Endpoint study record: Crystalline phase by XRD by NANOGENOTOX

Administrative Data

Purpose flag: key study (X) robust study summary ( ) used for classification ( ) used for MSDS

Study result type: experimental result

Data source

Reference

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
<th>Title</th>
<th>Testing laboratory</th>
<th>Report no.</th>
<th>Owner company</th>
<th>Company study no.</th>
<th>Report date</th>
</tr>
</thead>
<tbody>
<tr>
<td>study report</td>
<td>KA Jensen</td>
<td>2013</td>
<td>Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials</td>
<td>NRCWE (DK) and IMC-BAS (BG), LNE</td>
<td>D4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data access

other: owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Methods

x-ray diffraction (XRD)
Principles of method if other than guideline

X-Ray Diffraction (XRD) analysis is based on the principle that crystalline materials diffract X-rays in a characteristic pattern, which is unique for each material. XRD can therefore be used to identify different polymorphs, such as typical TiO2 polymorphs rutile, brookite and anatase. The width of the reflections can also give information about the size of the diffracting crystals (not necessarily the same as the particle size). XRD can be measured in different setups and different wavelengths are possible, but for standard measurements this is less important, as long as it is taken into account. Most databases are based on irradiation using Cu X-rays. The step length (if using Cu) is recommended to be 0.15. (Hill, 1986) All data presented in this report were recorded in reflection mode using Cu radiation, which is usually chosen for fast phase identification. Reflection mode analysis has the advantage that very small samples can be used (though more material is recommended) and the scatter is usually low until high values of 2theta, so unit cells can be determined with high accuracy. Internal standards are used to control for differences between instruments. XRD sizing limitations. As any method, sizing of crystallites by XRD has limitations. Most importantly, the method has both upper and lower limits, where the lower limit is very much material dependent. Large crystals have narrow reflections, and as rule of thumb, sizes cannot be calculated for crystals larger than 100 nm. As an example, using the first reflection from Anatase as starting point, and using the Scherrer Equation backwards, this gives the expected additional broadening of 0.014. Compared to the contribution from the instrument 0.072 from NRCWE and 0.097 from IMC-BAS, it is seen that the instrument contribution contributes most to the resulting peak. Another issue when calculating the crystal size from X-Ray diffraction is how accurate the results really are. At NRCWE it has been decided to round the sizes to whole numbers and list those as results; however for the comparison the numbers have been listed with one decimal. The real and important question is however; how accurate are the calculations? It is known that the larger the crystals get, the more the instrument contribution matters. However for very small crystals it is difficult to find the background and thereby the height of the reflection, so in this case it is also difficult to find the right FWHM, and calculate the right size. It was assumed that the results are more uncertain than we have listed. Our estimate is that the uncertainty probably is on the order of ±5 nm for all the samples.

Details on methods and data evaluation

Data treatment: Many programs are available for calculation on XRD data can directly calculate the crystal size. It can be quite difficult to find their actual way of calculation, but they are more or less based on the same principles of the Scherrer Equation, stating that the wider the reflections the smaller the crystals. NRCWE have chosen 2 types of software for calculations of the XRD data: 1. The Scherrer equation was used on data from “fityk”, a program only calculating the best fit for the reflections. 2. TOPAS, reporting both the size based on IB (integral breath) and FWHM (full width at half maximum). The crystal size was calculated by the Scherrer Equation. The width and position of the reflection has been found by using the program “fityk”. No structure is added in this program, it is merely calculating the best fit of the peak shape. The 0.89 K=shape factor value was used in the equation. Details of the data treatment, used softwares and data storage can be found in the attached file with the final report.

Data gathering

Instruments

The data from NRCWE were measured at room temperature (25°C) on a Bruker D8 Advanced Diffractrometer in reflection mode with Bragg-Brentano geometry. The analysis were made using Cu Kα1 X-rays (1.5406 Å) generated using a sealed Cu X-ray tube run at 40 kV and 40 mA. The x-ray beam was filtered for Cu Kα2 and focused using a primary beam Ge monochromator and fixed divergence slit 0.2°. The analyses were made in the stepping mode stepping 0.02 degree 2theta per second and data were collected using a linear PSD detector (Lynx-eye) with opening angle 3.3°.
The data from IMC-BAS were measured at room temperature (21°C) using a Bruker D2 Phaser diffractometer in reflection mode with θ-θ geometry. Cu X-rays were generated by a sealed Cu X-ray tube run at 30 kV and 10 mA and focused using a Ni filter and a fixed 0.2° divergence slit. Data generated with a step size of 0.02 degree 2θ and with a step time of 10 s and collected scintillation detector with opening angle 0.2°. Since the instrument does not use a monochromator, the raw data contains reflections from both Kα₁ and Kα₂ rays. For data comparison, the Kα₂ contribution was therefore stripped from the data using the EVA software (Bruker).

The data from LNE were measured on X’pert Pro MPD diffractometer. The X’pert Pro MPD diffractometer has a goniometer configuration θ-θ, which allows characterization of powders at high diffraction angles. LNE determined the association of Nickel filter, masks, slot and anti-scatter since these conditions leads to better results resolution / intensity spectrum exclusively for these analysis on specifics powders. The diffractograms were obtained with a scan on range of 2θ from 3 to 140°. The stepping of the goniometer was fixed for these tests to 0.03° for an acquisition time of 30 s. The chamber temperature was 25°C. Analyses were performed with Anode X-Ray tube Cu at 50kV and 35mA.

**Calibration**

The analysis were made usingCuKα1 X-rays (1.5406 Å) generated using a sealed Cu X-ray tube run at 40 kV and 40 mA. The x-raybeam was filtered for CuKα2 and focused using a primary beam Ge monochromator and fixed divergence slit 0.2°. The analyses were made in the stepping mode stepping 0.02 degree 2theta per second and data were collected using a linear PSD detector (Lynx-eye) with opening angle 3.3°.Each instrument has a unique contribution to the X-ray diffraction profile, which should bedocumented for detailed data comparisons using e.g., a large crystallite standard. For the analysis,NRCWE used a CeO2 (NIST SRM674a) standard. To assess the contribution from the instrument,the full width at half maximum, FWHM, was measured on the standard and plotted as a radianangle.

**Reproducibility**

**Test materials**

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

**Identifier** Reference Material/Nanomaterial

**Identity** NM-101

**State of test material**

other: fluffy powder

**Any other information on materials and methods incl. tables**

Many programs are available for calculation on XRD data can directly calculate the crystal size. It can be quite difficult to find their actual way of calculation, but they are more or less based on the same principles of the Scherrer Equation, stating that the wider the reflections the smaller the crystals (see below and in appendix).

At IMC-BAS the diffractogram were processed using three types of software:
1. Fullprof, freely available at http://www.ill.eu/sites/fullprof/;
2. TOPAS® application with the Bruker AXS®;
3. Winfit, a freeware that does not include Rietveld refinement, instead it uses a single or multi-peak fitting procedure and the Scherrer equation (4.1).

NRCWE have chosen 2 types of software for calculations of the XRD data:
1. The Scherrer equation was used on data from “fityk”, a program only calculating the best fit for the reflections.
2. TOPAS, reporting both the size based on IB (integral breath) and FWHM (full width at half maximum).

LNE performed their calculations according to the “Reference Intensity Ratio (RIR)”. The principle of this method is based on the determination of the intensity ratios between main peaks in relation to that of corundum in 50/50 mixture. RIR is recorded for rutile and anatase in the ICDD database (the International Centre for Diffraction Data). This method can be considered quantitative if there are only two main phases in the TiO₂ powder: e.g., anatase and rutile. The number of samples for analysis for each concentration must be at least 2 to estimate the repeatability of the measurement.

Results and discussions

Common name
NM 101 is anatase

Remarks on results including tables and figures

The Anatase samples, NM100, NM101, NM102 and NM105

Figure 0-3 The diffraction data from NRCWE and IMC-BAS. Overall the data are very alike. The long measurements are done at IMC-BAS and the short ones at NRCWE.
### Table 0-5 Crystallite sizes (nm) determined from measurements on NM101, Anatase

<table>
<thead>
<tr>
<th>Vial</th>
<th>LNE Scherrer Equation</th>
<th>IMC-BAS Peak fit, FWHM vs standard</th>
<th>Topas 4.2, standard less</th>
<th>Fullprof, quartz standard</th>
<th>NRCWE Scherrer Equation*</th>
<th>Topas 4.1, IB</th>
<th>Topas 4.1, FWHM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0180</td>
<td>no data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0239</td>
<td></td>
<td></td>
<td></td>
<td>7.0 ± 0.8</td>
<td>7.1 ± 0.2</td>
<td>10.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>0415</td>
<td></td>
<td></td>
<td></td>
<td>6.9 ± 0.7</td>
<td>7.7 ± 0.2</td>
<td>10.8 ± 0.2</td>
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</tr>
<tr>
<td>0510</td>
<td></td>
<td></td>
<td></td>
<td>7.2 ± 1.1</td>
<td>7.0 ± 0.1</td>
<td>9.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0729</td>
<td></td>
<td></td>
<td></td>
<td>6.5 ± 0.8</td>
<td>7.1 ± 0.1</td>
<td>9.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>1266</td>
<td>5.00&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.30&lt;sup&gt;f&lt;/sup&gt; (1.46)</td>
<td>6.84&lt;sup&gt;f&lt;/sup&gt; (0.95)</td>
<td></td>
<td></td>
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<tr>
<td>1268</td>
<td></td>
<td></td>
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<td>1270</td>
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<td></td>
</tr>
</tbody>
</table>

<sup>f</sup> Average of three samples; * Based on reflections: 101, 200, 105, 211, 116 and 220

### Table 0-6 Summary of XRD sizes calculated for TiO<sub>2</sub> using various instruments and principles.

<table>
<thead>
<tr>
<th>Supplier information</th>
<th>NM100&lt;sup&gt;e&lt;/sup&gt;</th>
<th>NM101</th>
<th>NM102</th>
<th>NM103</th>
<th>NM104</th>
<th>NM105 (Anatase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 - 220</td>
<td>57</td>
<td>5</td>
<td>18</td>
<td>-</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>IMC-BAS</td>
<td>62</td>
<td>5</td>
<td>16</td>
<td>19</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>IMC-BAS TOPAS</td>
<td>168</td>
<td>7</td>
<td>18</td>
<td>20</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>IMC-BAS Fullprof</td>
<td>&gt; 100</td>
<td>7</td>
<td>23</td>
<td>26</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>NRCWE Scherrer eq.</td>
<td>&gt; 100</td>
<td>7</td>
<td>26</td>
<td>25</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>NRCWE TOPAS, IB</td>
<td>&gt; 100</td>
<td>7</td>
<td>28</td>
<td>28</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>NRCWE TOPAS, FWHM</td>
<td>&gt; 100</td>
<td>10</td>
<td>28</td>
<td>28</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>LNE Scherrer eq.</td>
<td>141</td>
<td>-</td>
<td>30</td>
<td>18</td>
<td>23</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>e</sup> Size-data not reliable due to large crystallite size.

**Overall remarks, attachments**

**Attached full study report**


Remarks: Dispersion protocol


Remarks: Data in the report and details protocol in annex
Applicant's summary and conclusion

Conclusions
The calculated sizes from NRCWE are in all cases larger than those from IMC-BAS. This is ascribed to differences in instrumental performance and the calculation procedures used. However, almost all the differences can be covered by the estimated 5 nm real standard deviation in the analysis.

Cross-reference to other study

4.26 Crystallite and grain size

4.27 Aspect ratio/shape

4.28 Specific surface area

Endpoint study record: Specific surface area by INIA

Administrative Data
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source
Data access
other: performed and provided by INIA, Spian

Materials and methods
Methods
BET

Results and discussions
Specific surface area
Mean 289 m²/g
Standard deviation
Table: 3 Surface Area by BET

<table>
<thead>
<tr>
<th>NANOMATERIAL</th>
<th>Surface area (m²/g) from supplier</th>
<th>Surface area (m²/g) measured by BET</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂ P-25 EVONIK</td>
<td>35-65 (Value 57)</td>
<td>60</td>
</tr>
<tr>
<td>TiO₂ NM-105 (P25 Rutile-Anatase)</td>
<td>61</td>
<td>55</td>
</tr>
<tr>
<td>TiO₂ NM-104 (UV TITAN M-212)</td>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>TiO₂ NM-101 (HOMBIKAT)</td>
<td>&gt;250</td>
<td>289</td>
</tr>
<tr>
<td>TiO₂ NM-103 (UV TITAN M-612)</td>
<td>60</td>
<td>54</td>
</tr>
</tbody>
</table>

Overall remarks, attachments

Overall remarks
The surface area measured by BET (Table 3). The measured values are lower but very close to the information provided by the supplier.

Attached background material
table BET.doc

Endpoint study record: Specific surface area by SAXs by NANOGENOTOX

Administrative Data

Purpose flag  key study (X) robust study summary ( ) used for classification ( ) used for MSDS
Study result type  experimental result
Data source

Reference

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<th>study report</th>
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<tr>
<td>Author</td>
<td>KA Jensen</td>
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<tr>
<td>Year</td>
<td>2013</td>
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<td>Title</td>
<td>Deliverable 4.4: Determination of specific surface area of NANOGENOTOX nanomaterials</td>
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<td>Testing laboratory</td>
<td>CEA (F)</td>
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<td>Report no.</td>
<td>D4.4</td>
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Data access

other: owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Methods

other: SAXS and USAXS

Principles of method if other than guideline

Details of the method can be found in the attached SOP document.

Details on methods and data evaluation

Sample preparation: powder samples were prepared in 1.5 mm glass capillaries leading to typical equivalent thickness of dense material from 100 to 200 µm. The usual thickness of aqueous samples for SAXS measurement is 1 mm with an acquisition time of 1 hour. Dispersions for analysis are typically produced by sonication in a dispersion medium (see each dedicated SOP (general SOP from NANOGENOTOX) for specific dispersion protocols). The concentration required for analysis depends on the relative scattering length densities between particles and dispersion medium, and the density of materials. The sample must be stable within the time-frame of the measurement. Typical concentration in oxide for NANOGENOTOX suspensions is 3 g/L. Since the scattering length density of silica is relatively low, higher concentrations were used when possible. 2) Details on method Very detailed description of the method could be found in the attached SOP document. In order to calculate the sample transmission, the flux of incident and transmitted beam are measured and averaged over 200 s before running the SAXS measurement. The time of acquisition necessary for SAXS experiment depends on the sample properties. For TiO2 powders, two measurements were performed: one with a short time of 200 s or 150 s to get unsaturated data for small angles (low q), and one for a long time of 1800 s to get data in the high q region with low signal/noise ratio. For aqueous suspensions prepared for NANOGENOTOX, SAXS measurements were performed in kapton capillaries of internal thickness 1.425 mm and run for 3600 s, leading to transmissions of about 0.25. USAXS measurements were performed in 1 mm or 1.5 mm non-sticky double kapton cells. 3) Raw Data Treatment-Raw data, translated into intensity as a function of the scattering vector q, are first normalized by parameters of the experiments such as acquisition time, sample thickness and calibration constants determined using reference samples. -The data are thus expressed in
absolute scale (cm\(^{-1}\)). Backgrounds are then subtracted. SAXS data obtained for short time and long time are concatenated, together with USAXS data to get continuous diffractograms on the whole q range. For powder samples, the Porod law is applied to extract specific surface areas of raw materials. Data from suspensions are fitted with a model describing fractal aggregates of primary particles. In this model, the whole q range is divided into sections reflecting different structural levels in the sample, and fitted by local Porod and Guinier scattering regimes. Intensity average parameters are then determined such as radius of gyration for the primaries and for the aggregates, and a fractal dimension for the aggregates. Invariants are calculated, which give a correlation between the sample concentration and the specific surface area obtained in suspension.

4) SSA from SAXS
Specific surface area determination from SAXS on powders
To treat raw SAXS data and get absolute intensities, the intensity by the thickness of the scattering material need to be normalised. However, for powder samples, the sample thickness is not well defined and cannot be precisely controlled as it depends on the powder compaction and the different scales of porosity. To elude this problem, a model system is used, considering the effective thickness of material crossed by X-rays, called eB, corresponding to an equivalent thickness if all the material would be arranged in a fully dense (no inner or outer porosity) and uniform layer. Details of the method can be found in the attached file with SOP.

**Used Protocols**
The attached protocol describes the general procedure applied at CEA/LIONS (Laboratoire Interdisciplinaire sur l'Organisation Nanométrique et Supramoléculaire) to perform Small Angle X-ray Scattering measurements and to treat the data to extract physical-chemical properties of materials. This procedure was applied in the framework of NANOGENOTOX among others to characterize SiO\(_2\) manufactured nanomaterials as raw powders and SiO\(_2\) in aqueous suspensions.

**Used Protocols: attached files**
- **Attached document** SOP_SAXS_CEA.doc / 2.38 MB (application/msword): SIAR, ENV/JM/MONO(2015)17/PART1/ANN1

**Remarks**
Protocol for SAXS measurements in CEA laboratories

**Data gathering**

**Instruments**
The main set up components used for SAXS and USAXS experiments at CEA/LIONS:
- X-ray generator: Rigaku generator RUH3000 with copper rotating anode (\(\lambda = 1.54\) Å), 3kW
- Home made optic pathways and sample holders (with two channel-cut Ge (111) crystals in Bonse/Hart geometry for USAXS set up, cf Lambard (1992).
- Flux measurement for SAXS set up: pico amperemeter Keithley 615
- Flux measurement for USAXS set up: DonPhysik ionization chamber
- Detector for SAXS set up: 2D image plate detector MAR300
- Detector for USAXS set up: 1D high count rate CyberStar X200 associated to a scintillator/photomultiplier detector.

All experimental parameters are monitored by computer by a centralized control-command system based on TANGO, and interfaced by Python programming. 2D images are treated using the software ImageJ supplemented with some specific plugging developed at CEA/LIONS. This control-command system has been achieved by Olivier Taché and is detailed in: O. Taché ; « Une architecture pour un système évolutif de contrôle commande d'expériences de physique », Engineer thesis, 2006, available at http://tramis.cea.fr/sis2m/lions/tango/tango-ds/memoire.pdf

**Calibration**
- A sample of 3 mm of Lupolen® (semi crystalline polymer) was used for the calibration of the intensity in absolute scale, the maximum intensity being adjusted to 6 cm\(^{-1}\). A sample of 1 mm of octadecanol was used for the calibration of the q range (calculation of sample-to-detector distance), the position of the first peak standing at 0.1525 Å\(^{-1}\). Calibrations in intensity and in q range were performed before each
series of measurements.

**Test materials**

Test material equivalent to submission substance identity

yes

**Reference Material/Nanomaterial and Sample identification number**

**Identifier**  Reference Material/Nanomaterial

**Identity**  NM-101

**State of test material**

other: fluffy powder

**Confidential details on test material**

Commercial name:

**Results and discussions**

*Table 2: Specific surface area results for TiO$_2$ powders from SAXS measurements.*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lim Iq$^4$ [10$^{-3}$ cm$^{-1}$]</th>
<th>$\Sigma$ [m$^{-1}$]</th>
<th>Specific surface area [m$^{2}$/g]</th>
<th>error on plateau</th>
<th>$+$ 5% error on density</th>
<th>Equivalent diameter for spheres [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM101</td>
<td>52.7</td>
<td>7.17E+08</td>
<td>169.5</td>
<td>+/-8.5</td>
<td>+/-25.4</td>
<td>8</td>
</tr>
</tbody>
</table>

All SAXS diffractograms and the corresponding representations in I(q)q$^4$ for TiO$_2$ NM powders are displayed in Figure 4.1, 4.2 and 4.3. Figure 4.2 shows the I(q)q$^4$ representation and Porod’s plateaus raw-plots for each of the TiO$_2$ NM.
Figure 4.1: SAXS and USAXS results for TiO$_2$ raw powders NM101 (blue crosses), NM102 (green circles), NM103 (red triangles), NM104 (blue diamonds) and NM105 (pink square).

Figure 4.2: Representation in $Iq^4$ of SAXS and USAXS results of NM101 (blue crosses), NM102 (green circles), NM103 (red triangles), NM104 (blue diamonds) and NM105 (pink squares). The dotted lines are the corresponding Porod's plateaus.

Figure 4.3: SAXS and USAXS results for TiO$_2$ raw powders of a) NM101;

Specific surface area

Mean 169.5 m$^2$/g

Standard deviation 8.5 m$^2$/g

Overall remarks, attachments

Attached full study report

Attached document D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1
Endpoint study record: Specific surface area by BET by NANOGENOTOX

Materials and methods

Methods

BET

Principles of method if other than guideline

Surface area and porosity are important characteristics, in understanding the structure, formation and potential applications of different natural materials. For this reason it is important to determine and control them accurately. The most widely used technique for estimating surface area is the so-called BET method (Brunauer, Emmett and Teller, 1938) [5]. The concept of the theory is an extension of the Langmuir theory, which is a theory for monolayer molecular adsorption, to multilayer adsorption with the following hypotheses: (a) gas molecules physically adsorb on a solid inlayers infinitely; (b) there is no interaction between each adsorption layer; and (c) the Langmuir theory can be applied to each layer.
Details on methods and data evaluation

BET analyzer operates by measuring the quantity of gas adsorbed onto or desorbed from a solid surface at some equilibrium vapor pressure. The data are obtained by admitting or removing a known quantity of adsorbate gas (Nitrogen) into or out of a sample cell containing the solid adsorbent maintained at a constant temperature below the critical temperature of the adsorbate (at temperature of liquid Nitrogen). As adsorption or desorption occurs the pressure in the sample cell changes until equilibrium is established. The quantity of gas adsorbed or desorbed at the equilibrium pressure is the difference between the amount of gas admitted or removed and the amount required to fill the space around the adsorbent (void space). Sample preparation requires no special treatment. Measurements performed on powder. 0.1 g of the material placed in the appropriate cell size (the volume of the sample may vary from sample to sample due to difference in density etc.). Details of the method and values of used parameters might be found in the attached file with full study report: Draft D4.4_specific surface area.

Data gathering

Instruments

High-speed surface area and pore size analyzer NOVA 4200e (Quantachrome). NOVA 4200e equipped with four preparation ports (vacuum or flow degassing) and four analysis ports. It provides single and multi-point BET surface area with y-intercept, "C" constant, slope and correlation coefficient; up to 100 adsorption and 100 desorption isotherm points; B.J.H pore size distribution calculated from the adsorption or desorption isotherm; total pore volume and average pore radius.

Reproducibility

two measurements were performed

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Reference Material/Nanomaterial</th>
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<tbody>
<tr>
<td>Identity</td>
<td>NM-101</td>
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Test material identity

<table>
<thead>
<tr>
<th>Identifier</th>
<th>CAS number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>7631-86-9</td>
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State of test material

other: fluffy powder

Any other information on materials and methods incl. tables

The results from the BET analyses conducted in the project was compared with manufacturers data. BET (manufacturer) (m²/g): > 250
Results and discussions

Specific surface area

<table>
<thead>
<tr>
<th>Material</th>
<th>BET surface</th>
<th>Total pore volume</th>
<th>Micro surface area</th>
<th>Micropore volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m²/g</td>
<td>ml/g</td>
<td>m²/g</td>
<td>ml/g</td>
</tr>
<tr>
<td>NM101</td>
<td>316.07</td>
<td>0.3190</td>
<td>13.625</td>
<td>0.00179</td>
</tr>
</tbody>
</table>

Remarks on results including tables and figures

Figure 4.10: Isotherms of nitrogen sorption experiments at 77K for the TiO₂ nanomaterials

Overall remarks, attachments

Attached full study report

Attached document  D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1

Remarks  Dispersion protocol


Remarks  Data in the report and details protocol in annex

Applicant's summary and conclusion

Conclusions

see the endpoint: comparison between BET and SAXS

Cross-reference to other study

Endpoint study record: Specific surface area comparison between SAXS and BET results by NANOGENOTOX

Administrative Data

Purpose flag  key study ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type  experimental result

Reference

<table>
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<tr>
<th>Reference type</th>
<th>study report</th>
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<tr>
<td>Author</td>
<td>KA Jensen</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 4.4: Determination of specific surface area of NANOGENOTOX nanomaterials</td>
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<td>Testing laboratory</td>
<td>IMC-BAS (BG) AND CEA</td>
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<tr>
<td>Report no.</td>
<td>D4.4</td>
</tr>
<tr>
<td>Company study no.</td>
<td></td>
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<tr>
<td>Report date</td>
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Data source

Data access
other: owner: NANOGENOTOX

Materials and methods

Attached document  D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks  Dispersion protocol

Remarks  Data in the report and details protocol in annex

Principles of method if other than guideline
see the report

Results and discussions

Table 7: Summary of the specific surface area data obtained by BET and SAXS

<table>
<thead>
<tr>
<th>Material</th>
<th>BET specific surface area m²/g</th>
<th>SAXS surface m²/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnO</td>
<td>10.037</td>
<td></td>
</tr>
<tr>
<td>TiO₂</td>
<td>169.5(8.5)</td>
<td></td>
</tr>
<tr>
<td>NM101</td>
<td>316.07</td>
<td></td>
</tr>
</tbody>
</table>
**Applicant's summary and conclusion**

**Conclusions**

Comparison between SAXS and BET results. The results from both analytical methods show a difference for NM101 material. BET specific surface area (m²/g): 316.07, SAXS specific surface area (m²/g): 169.5. Assessed from the methodology, most of the differences may be explained by the combined errors in density and placement of plateau. Other explanations may come from the difference in thermal treatment and outgassing of the powders before BET analysis.

**Executive summary**

The samples were analyzed for their specific surface area using BET and SAXS, which are two different analytical methods relying on nitrogen gas adsorption and X-ray scattering, respectively. Proof of principle has been shown for SAXS analysis of all three compounds (TiO₂ amorphous silica and CNT) for the deduction of surface area is applicable. However, there is not an overall linear correlation between SAXS and BET data. The SAXS appears to underscore the specific surface area determined by BET. In this assessment, one must also consider the differences and limits of the methods. The determination of surface area for very small and bigger (>200 nm) particles needs more attention. The BET results given by producers are generally in very good agreement with the NANOGENOTOX data. This suggests that producer instrumental capacity and the SOPs for making BET analysis are similar or of same quality as the procedures used in NANOGENOTOX. All being well as SAXS data confirms the obtained results.

**4.29 Zeta potential**

**Endpoint study record: Zeta potential by INIA**

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Data source**

**Data access**
other: performed and provided by INIA, Spian

**Materials and methods**

**Methods**
other: DLS

**Used Protocols**

The Nanocare Protocol has been used for the preparation of aqueous suspensions of Titanium dioxide nanoparticles for six different concentrations and five different nanomaterials. Their aggregation/agglomeration has been measured by DLS (Zetasizer Nano Series, Malvern Instruments ZEN 3600), and their stability has been followed up for 14 days. Stocks suspension: Mass concentration: 1mg/L, 10 mg/L, 50 mg/L and 100mg/L. Solvent: Milli-Q water. The stock suspension was stirred at 900 rpm for 24 h at room temperature in a glass bottle wrapped with aluminium foil. Conditions: Glass Bottle (1L, borosilicate 3.3) Diluted suspensions: Mass concentration: 0.1mg/L and 0.01 mg/L.
Data gathering

Test materials

State of test material
dispersion

Results and discussions

The zeta potential, measured in water, shows for NM-101 particles a negative zeta potential across all concentrations tested. In general, concentrated dispersions appeared to have more stable aggregates than diluted suspensions.

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>P-25 Evonik Mean (mV)</th>
<th>NM-105 Mean (mV)</th>
<th>NM-101 Mean (mV)</th>
<th>NM-103 Mean (mV)</th>
<th>NM-104 Mean (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>-25.3</td>
<td>5.30</td>
<td>-28.3</td>
<td>--</td>
<td>10.6</td>
</tr>
<tr>
<td>50</td>
<td>-27.0</td>
<td>-16.6</td>
<td>-25.4</td>
<td>18</td>
<td>18.5</td>
</tr>
<tr>
<td>1</td>
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<td>-27.7</td>
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<td>-7.71</td>
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<tr>
<td>0.1</td>
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<td>-19.7</td>
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<tr>
<td>0.01</td>
<td>-16.3</td>
<td>-31.4</td>
<td>-10.2</td>
<td>-24.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>P-25 Evonik Mean (mS/cm)</th>
<th>NM-105 Mean (mS/cm)</th>
<th>NM-101 Mean (mS/cm)</th>
<th>NM-103 Mean (mS/cm)</th>
<th>NM-104 Mean (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.01150</td>
<td>0.02130</td>
<td>0.02320</td>
<td>--</td>
<td>0.00848</td>
</tr>
<tr>
<td>50</td>
<td>0.02890</td>
<td>0.02150</td>
<td>0.02440</td>
<td>0.00634</td>
<td>0.00912</td>
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<td>1</td>
<td>0.01640</td>
<td>0.01390</td>
<td>0.04480</td>
<td>0.01110</td>
<td>0.02430</td>
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<tr>
<td>0.1</td>
<td>0.01410</td>
<td>0.00606</td>
<td>0.01170</td>
<td>0.00621</td>
<td>0.02710</td>
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<td>0.01</td>
<td>0.00406</td>
<td>0.00654</td>
<td>0.00989</td>
<td>0.01820</td>
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Overall remarks, attachments

Overall remarks
Endpoint study record: Zeta potential by University of Graz

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS

Study result type experimental result

Data source

Data access

other: performed and provided by University of Graz

Materials and methods

Details on methods and data evaluation

As solid particles show low stability and a high tendency to aggregate in aqueous dispersions, our first goal was to produce a stable TiO₂ dispersion. Several pre-tests had been carried out with sample ID NM-105. These tests included coatings with sodium citrate and lecithin (as TiO₂ particles are known to be lecithin coated in sunscreen), as well as different sonication methods. Furthermore the effects of pH and ionic strength on the surface charge of the particles were investigated. The particles were characterized in terms of their physico-chemical properties (i.e., i. size, ii. distribution, iii. agglomeration, iv. surface charge) with Photon Correlation Spectroscopy (PCS) using a ZetaSizer Nano-ZS (Malvern).

Results and discussions

Sponsorship Program: Titanium Dioxide Report

Karl Franzens University Graz, Institute of Pharmaceutical Sciences, Pharmaceutical Technology, Dr. Eva Roblegg and Sandra Blass

Results of the particle characterization of NM101 in different biological media

<table>
<thead>
<tr>
<th>NM101</th>
<th>0.4 mg/ml TiO₂ particles (NM101, 7nm, anatase, Fa. Hombikat) untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Size (d.nm)</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>1609</td>
</tr>
<tr>
<td>PBS</td>
<td>1188*/5148</td>
</tr>
<tr>
<td>DMEM + L-Glutamine</td>
<td>1438*/5560</td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>1201*/5232</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>1278</td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>1406</td>
</tr>
</tbody>
</table>

| 0.4 mg/ml TiO₂ Partikel (NM101, 7nm, anatase, Fa. Hombikat) 1 min Sonifier (40% Amplitude) |
|-------|---------|---------|---------|---------|---------|
| Medium | Size (d.nm) | Z-Average (d.nm) | PdI | Zeta Potential (mV) | monomodal | Zeta Deviation |
| MQ Wasser | 719,5 | 500,9 | 0,274 | -27,2 | 5,5 |
| PBS | 2254 | 1827 | 0,283 | -19,7 | 16 |
| DMEM + L-Glutamine | 2854 | 2350 | 0,217 | 22,3*/-34,3/-92,0 | -5,52 | 43,7 |
Results of the particle characterization with a Mastersizer 2000

<table>
<thead>
<tr>
<th>Medium</th>
<th>Size (0.1) [nm]</th>
<th>Size (0.5) [nm]</th>
<th>Size (0.9) [nm]</th>
<th>Sonification period</th>
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<tbody>
<tr>
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<td>16609</td>
<td>986132</td>
<td>1601490</td>
<td>0 min US</td>
</tr>
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<td>630</td>
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<td>13492</td>
<td>1 min US</td>
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<td>655</td>
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<td>2 min US</td>
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<td>MQ Wasser</td>
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<td>879</td>
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Overall remarks, attachments

Attached background material

Uni Graz_Roblegg_Agglomeration NM101.docx
4.30 Surface chemistry

4.31 Dustiness

Endpoint study record: Dustiness by Small Rotating Drum (SD) method by NANOGENOTOX

Administrative Data

Purpose flag  key study (X) robust study summary ( ) used for classification ( ) used for MSDS
Study result type  experimental result

Data source

Reference

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<th>Reference type</th>
<th>Author</th>
<th>Year</th>
<th>Title</th>
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<td>study report</td>
<td>KA Jensen</td>
<td>2013</td>
<td>Deliverable 4.6: Dustiness of NANOGENOTOX nanomaterials using the NRCWE small rotating drum and the INRS Vortex shaker</td>
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Bibliographic source

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<th>Report no.</th>
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<tr>
<td>NRCWE (DK)</td>
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Data access

other: owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Methods

other: Small Rotating Drum method

Principles of method if other than guideline

The small rotating drum was designed as a downscaled version of the EN 15051 rotating drum while maintaining important test parameters.

Details on methods and data evaluation

- The small rotating drum was designed as a downscaled version of the EN 15051 rotating drum while maintaining important test parameters. This enabled testing of smaller material amounts (~6g instead of ~500g).
- The drum consists of a cylindrical part [internal diameter (i.d.) 16.3 cm, length 23.0 cm, volume 4.80 l] with a truncated cone at each end (half angle 45°, length 6.3 cm, volume 1.13 l). The total volume of the drum is 5.93 l.
- The drum was made of stainless steel and all inside surfaces were polished to 450 ± 50 gloss units to minimize surface adhesion and to facilitate cleaning.
- The drum was electrically
grounded as prescribed by EN 15051. The drum contains three lifter vanes (2 x 22.5 cm). In EN 15051, a 1-min rotation at 4 rpm and eight lifter vanes are prescribed. Therefore, the present drum was operated at 11 rpm to obtain the same number of powder parcels falling per minute as in the EN 15051 test (Schneider and Jensen, 2008). The inlet air to the drum was controlled at 50 % RH and HEPA-filtered to ensure no particle background. In the applied set-up, respirable dust is collected by a GK2.69 respirable dust sampler at 4.2 lpm (BGI, UK) and dust particle size-distributions are measured using the Fast Mobility Particle Sizer (FMPS 3091, TSI), with a range of 5.6 to 560 nm, and the Aerodynamic Particle Sizer (APS 3321, TSI) with a range of 0.5 to 20 μm. It is important to note that these two instruments provide a size distribution which is expressed for the FMPS in electric mobility equivalent diameter, whereas for the APS, it is the equivalent aerodynamic diameter that is obtained. A GRIMM CPC may be connected for simultaneous number-concentration measurements, but not used in this study. The dustiness test was conducted in triplicates for each powder preceded by a so-called saturation run completed to coat all inner surfaces of the system with dust. The saturation test was performed using 2 grams of powder and rotation for 60 seconds. Then the actual triplicate tests were completed using 6 grams of test material per run. After each run the drum was emptied by pouring out the residual powder and gently tapping the drum three times with a rubber hammer. When loading the powder in the drum, it was carefully placed centrally in the drum on the upwards moving side of three inner lifter vanes placed at bottom position. Then the drum was sealed followed by 60 seconds of background measurements were done to ensure a particle free test atmosphere. The experiment was then initiated by rotating the drum for 60 seconds during which particles were emitted and led through the airflow to the sampling train. After the drum was stopped, measurements and sampling was continued for additional 120 sec to catch the remaining airborne particles in the dust cloud. Thus, the total time during which the measurement is made is 180 s. This then completed the rotational test. The drum and sampling lines were thoroughly cleaned between each powder type using a HEPA-filter vacuum cleaner designed for asbestos cleaning and wet-wiping. Then the drum was let to air-dry before the next powder could be tested. The mass of collected respirable dust was determined after conditioning the filters and controls in our weighing room (22°C; 50 %RH) using a Sartorius microbalance (Type R162 P; Sartorius GmbH, Göttingen, Germany). The mass is used to categorize the dustiness levels of the powders according to EN15051. Additional information may be found in the attached detailed final report on dustiness measurements.

Data gathering

Instruments

In the applied set-up, respirable dust is collected by a GK2.69 respirable dust sampler at 4.2 lpm (BGI, UK) and dust particle size-distributions are measured using the Fast Mobility Particle Sizer (FMPS 3091, TSI), with a range of 5.6 to 560 nm, and the Aerodynamic Particle Sizer (APS 3321, TSI) with a range of 0.5 to 20 μm. It is important to note that these two instruments provide a size distribution which is expressed for the FMPS in electric mobility equivalent diameter, whereas for the APS, it is the equivalent aerodynamic diameter that is obtained. A GRIMM CPC may be connected for simultaneous number-concentration measurements, but not used in this study.

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM-101
State of test material
other: fluffy powder

Results and discussions

Number of dust particles and mass-based dustiness indexes of TiO2 nanomaterials (NM10x) and SAS (NM20x) nanomaterials. Experimental data with the SD method are obtained over a test time of 180 s as explained in the chapter 2.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Test mass (g)</th>
<th>Number (1/mg) CPC</th>
<th>Dustiness index</th>
<th>Mass (mg/kg)</th>
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<td>NM-101</td>
<td>6</td>
<td>1.10E+06</td>
<td>728 (±10)</td>
<td>24 (±9)</td>
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Remarks on results including tables and figures
Figure 4 shows the particle number size distributions of aerosols generated during rotating drum dustiness testing of the TiO2 samples. It is evident that all TiO2 powders appear to generate fine aerosol with an electrical mobility equivalent peak diameter typically between 200 and 250 nm. Larger µm-size-modes are present all in samples. One sample, NM-102, was very dusty and generated slightly higher concentrations of µm-size dust particles than sub-µm size dust particles. This is an unusual particle size-distribution profile.

Figure 4: Particle number size distributions for TiO2 MN obtained with the SD method. All distributions are presented as given by the FMPS (electrical mobility equivalent diameter) and APS (aerodynamic equivalent diameter).

Error! Reference source not found. shows the particle number size distributions of aerosols generated during rotating drum dustiness testing of the SAS MN samples. The SAS powders generate fine aerosol with an electrical mobility equivalent peak diameter typically between 200 and 300 nm. Larger µm-size-modes are present in all samples, but none of the coarse mode particle concentrations exceed the 200-300 nm mode-size particle concentrations. NM-203 produces the lowest number of particles, but also generates
the finest dust particles measured by FMPS as seen by smallest peak sizes and a shoulder mode around 60 nm.

**Figure 5:** Particle number size distributions for SAS MN. All distributions are presented as given by the FMPS (electrical mobility equivalent diameter) and APS (aerodynamic equivalent diameter).

Figure 6 and Figure 7 shows respectively the dustiness ranking of inhalable and respirable dust for TiO$_2$ nanomaterials (NM10x) and SAS (NM20x). Compared to conventional mass-based dustiness indexing of the EN 15051 standard, the TiO$_2$ nanomaterials vary from low to high dustiness in both size fractions. There also seems to be good agreement between the respirable and inhalable dustiness indexing. Concerning the SAS nanomaterials, the index are only categorized with high dustiness indices. However, there appears to be a larger variation in respirable dustiness ranking than for inhalable dust where NM-204 is observed to have very high dustiness levels.
Figure 6: Dustiness ranking of inhalable dust for TiO2 nanomaterials (NM10x) and SAS (NM20x) nanomaterials as obtained with the small rotating drum method at NRCWE.

![Respirable Dustiness Index](image)

Figure 7: Dustiness ranking of respirable dust for TiO2 nanomaterials (NM10x) and SAS (NM20x) nanomaterials as obtained with the small rotating drum method at NRCWE.

Overall remarks, attachments

Overall remarks

The powder generate fine aerosol with an electrical mobility equivalent peak diameter typically between 200 and 300 nm. Larger μm-size-modes are present in all samples, but none of the coarse mode particle concentrations exceed the 200-300 nm mode-size particle concentrations.

Attached full study report

Attached document D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks Dispersion protocol

Remarks Data in the report and detailes porocol in annex

Applicant's summary and conclusion

Cross-reference to other study

Endpoint study record: Dustiness by Vortex Shaker (VS) method by NANOGENOTOX

Administrative Data

Purpose flag key study (X) robust study summary ( ) used for classification ( ) used for MSDS
Study result type  experimental result

Data source

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<tr>
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Data access

Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Details on methods and data evaluation

Vortex Shaker (VS) method

The vortex shaker method (VS) consists of a centrifuge stainless tube agitated by a vortex in which the testpowdered material is placed together with 100 μm diameter bronze beads. These are used to help the deagglomeration of powders. HEPA filtered air, controlled at 50% RH, pass through the tube in order to transfer the released aerosol to the sampling and measurement section. The protocol developed for the experiments performed within this project used two different versions of the sampling and measurement section. All tests were conducted with VS method using approximately 0.5 ml powder, which is placed in the sample vialtogether with 5 g bronze beads (100 μm), used to agitate and deagglomerate the powder. The sample is allowed conditioning in the 50% RH before the shaker for a powder agitation period of 3600 s (60 min). Two different setup version were developed. The first version is devoted for real-time measurement using ELPITM Classic (10 Lpm, Dekati) for size distributions according to the equivalent aerodynamic diameter and CPC (Model 3786 UWCPC, TSI) for number concentrations. This version is also devoted for collecting airborne particles for subsequent electron microscopy (EM) observations. The test on the sample have been performed three times with this setup. The results of the tests performed with this first version of the VS method leads to the determination of:

- Dustiness indices expressed as the total number of particles emitted (based on data from CPC).
- Particle size-distribution of the aerosol (based on data from ELPITM Classic in its standard configuration).

The CPC used was the Model 3785 Water-based Condensation Particle Counter (TSI, USA). This CPC detects particles from 5 to >3000 nm. It provides a wide, dynamic, particle-concentration range, an essential characteristic for the tests considered. Featuring a single-particle-counting mode with continuous, live-time coincidence correction and a photometric mode, the CPC measures particle number concentrations up to 107 particles/cm3 with high accuracy. ELPITM (Electrical Low Pressure Impactor) is an instrument to measure airborne particle size distribution and concentration in real-time. It operates in the size range of 7 nm – 10 μm in its standard configuration. Because of its wide particle size range and rapid response (< 5 s), the ELPITM has been considered an ideal measurement instrument for the analysis of the unstable concentrations and size distributions, or the evolution of size distributions that could be
observed in these tests. In order to prevent particle bounce and charge transfer during the tests, all collection substrates used (PVC GELMAN GLA-5000 5μm / 25 mm) have been greased. In the ELPI the measured current signals are converted to (aerodynamic) size distribution using particle size dependent relations describing the properties of the charger, the impactor stages, and the effective density of the particles. The particle effective density provides a relationship between mobility and aerodynamics sizes. Effective density is a parameter which is complex to measure (Olferta et al., 2007), and values for samples used in the project are not available in the literature. Therefore, the following assumption has been made for the data from the ELPI: spherical particle with a density equal to the density of the condensed phase of the material constituting the NM. Density used for NM 200 was: 2.2 g/cm³ based on Kim et al. (2009). If this assumption is questionable, there is no robust method that can be applied to polydisperse aerosols over a wide size range, such as those used in the project. However, to assess the effect of this parameter on the results, the number size distributions were also calculated for a density of 1 g/cm³. The details of the calculation can be found in the attached file with the full report. To get information on particle morphology of the emitted aerosol, a simple but specific sampling set-up has been designed (see attached file with the full report). Transmission electron microscope (TEM) copper grids were taped onto 25 mm diameter polycarbonate membrane filters (0.4 or 0.8μm). Fiber backing filters were used to support the polycarbonate filters. Air flow was driven by a personal sampling pump at a flow rate of 1 L/min. The duration of the sampling has been set to 1 hour. The sampling period was set equal to the duration of a test (1 hour). For some tests, the sample was accumulated over two trials in order to have enough particles to observe. Different TEM copper grids having different carbon have been used (Carbon film, Quantifoil Holey Carbon Films or Holey Carbon Support Film). It is important to note that the duration of the test is to be considered as the process is dynamic. In the original INRS protocol developed, the duration of a test was set equal to 3600 s. But in the first version of the set-up as the instruments measure in real time, it is possible to perform the calculation for different durations between 0 and 3600 s. In this report, the calculations based on the CNC data were performed for two durations: 180 s and 3600 s. The first duration (180 s) was chosen to be consistent with the method SD. For the second version of the setup, the duration of the test was set to 3600 s, which corresponds to the original protocol of the Vs method. The second version of the setup is used for collecting respirable mass fraction of the emitted aerosol. The respirable mass fraction is obtained by sampling with a GK2.69 cyclone (BGI, UK). The filters have been preweighed and post-weighed following the recommendations of the ISO 15767:2009 on the same analytical balance. Only one test was performed with this setup due to time constraints. This is why the results are not presented with a confidence interval based on reproducibility. However, measurement uncertainty has been calculated for each measurement performed. The dustiness index in respirable mass (mg) of particles per kilogram, , was calculated as the respirable mass of generated particles in milligrams divided by the total mass of the test NM sample in kilograms: The recommendations of the standard ISO 15767:2009 were followed to determine the LOD of the weighing procedure for the filters used for sampling respirable mass of particles during this project. The LOD for the PVC GELMAN GLA-5000 (5 μm/37 mm) filters was equal to 20 ng. This value is used to determine the LOD expressed in dustiness index. The flow diagram of the experimental protocol used for the NGT project can be found in the attached file with full study report. The preparation of NM samples for VS testing include: 1) to take a series of 7 samples of 0.5 cm³ of the vial containing the nanomaterial received at the laboratory in this project, 2) to accurately weigh the samples. Three of the samples are devoted for test with the first version of the set-up (real-time measurement), one for the second version (respirable mass fraction measurement,) and three for the gravimetric water content measurement. Any additional samples are intended to further testing that would be needed in case of default validation. Microcentrifuge graduated tubes with secure seals and caps have been chosen to keep the 0.5 cm³ samples. The gravimetric water content was performed using a HR83 Halogen Moisture Analyzer (Mettler Toledo) and following a drying program defined specifically for small quantities of used NM (Temperature = 160°C; duration = 170 s). The weighing of the NM samples was performed with a XP205 analytical balance (10 μg readability, Mettler Toledo) while the weighing of the 37-mm filters from the respirable sampler was performed with a MX5 microbalance (1 μg readability, Mettler Toledo). Particular attention was given to the experimental device cleaning between successive
tests. All pipes and other connections were systematically cleaned with water and/or ethanol and dried in an oven, or eventually changed. The checking of the airflows was performed using a primary flow bubble calibrator (Gillian® Gillibrator 2). Prior to each test, the cleanliness of the air was assessed on the basis of measurements made using the CNC. In the case of a non-compliant result, everything was taken from the beginning. The validation of a test depends on several factors such as: 1) the stability of the parameters during the test, 2) a good reproducibility of measured number concentrations, 3) a good sequence of steps for the respirable aerosol sampling etc. The entire set-up was located inside a variable volume fume hood to prevent exposure of the operator. Similarly, all operations like weighing, water content measurement and sample preparation were carried out in a specific containment system that has a unique turbulent-free, low flow design which allows sensitive balance to operate without fluctuation and protects the operator from exposure to airborne particles that could be released when handling and weighing NM samples.

**Used Protocols**

The recommendations of the standard ISO 15767:2009 were followed to determine the LOD of the weighing procedure for the filters used for sampling respirable mass of particles during this project.

**Data gathering**

**Instruments**

1st setup: ELPITM Classic (10 Lpm, Dekati) for size distributions CPC (Model 3786 UWCPC, TSI) for number concentrations substrates used PVC GELMAN GLA-5000 5μm / 25 mm Different TEM copper grids having different carbon have been used (Carbon film, Quantifoil Holey Carbon Films or Holey Carbon Support Film). TEM not specified

Second setup: The respirable mass fraction is obtained by sampling with a GK2.69 cyclone (BGI, UK). The gravimetric water content was performed using a HR83 Halogen Moisture Analyzer (Mettler Toledo). The checking of the airflows was performed using a primary flow bubble calibrator (Gillian® Gillibrator 2)

**Calibration**

Particular attention was given to the experimental device cleaning between successive tests. All pipes and other connections were systematically cleaned with water and/or ethanol and dried in an oven, or eventually changed. The checking of the airflows was performed using a primary flow bubble calibrator (Gillian® Gillibrator 2). Prior to each test, the cleanliness of the air was assessed on the basis of measurements made using the CNC. In the case of a non-compliant result, everything was taken from the beginning. The validation of a test depends on several factors such as: 1) the stability of the parameters during the test, 2) a good reproducibility of measured number concentrations, 3) a good sequence of steps for the respirable aerosol sampling etc. The entire set-up was located inside a variable volume fume hood to prevent exposure of the operator. Similarly, all operations like weighing, water content measurement and sample preparation were carried out in a specific containment system that has a unique turbulent-free, low flow design which allows sensitive balance to operate without fluctuation and protects the operator from exposure to airborne particles that could be released when handling and weighing NM samples.

**Compliance with standard (ISO/CEN/other)**

yes The recommendations of the standard ISO 15767:2009 were followed to determine the LOD of the weighing procedure for the filters used for sampling respirable mass of particles during this project

**Test materials**

**Reference Material/Nanomaterial and Sample identification number**

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<thead>
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<td>Identity</td>
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Test material identity

Identifier   CAS number
Identity     7631-86-9

Results and discussions

Table 1: Gravimetric water content and bulk density of the TiO$_2$ and SiO$_2$ samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample mass (mg)</th>
<th>Water content (wt % dry)</th>
<th>Bulk density (g/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM-101</td>
<td>110</td>
<td>10%</td>
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</table>

Number-based data with the VS method are calculated from the time profiles with two test times (see page Error! Bookmark not defined.): 180 s and 3600 s. The first duration (180 s) was chosen to correspond to the test duration of the SD method. The mass-based data correspond to the respirable fraction only as the inhalable fraction was not part of the VS original protocol. The duration for the mass-based data is 3600 s.

Table 2: Number-based and mass-based dustiness indexes of TiO$_2$ nanomaterials (NM101),

<table>
<thead>
<tr>
<th>Sample</th>
<th>Test mass (mg)</th>
<th>CPC (S.D)$^b$</th>
<th>ELPI$^a$ (S.D)$^b$</th>
<th>CPC (S.D)$^b$</th>
<th>Respirable (S.D)$^c$</th>
<th>Mass (mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>NM-101</td>
<td>206.6</td>
<td>1.6E+04</td>
<td>(2.1E+04)</td>
<td>3.2E+05</td>
<td>(7.5E+04)</td>
<td>3.1E+06</td>
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</table>

$^a$ The assumption for calculating the number of particles emitted from the data from the ELPI is: spherical particle with a density equal to the density of the condensed phase of the material constituting the NM. Densities used were: 3.84 g/cm$^3$ for NM100, 101, 102 and 4.26 g/cm$^3$ for NM103, 104, 105 based on Teleki et al. (2008); 2.2 g/cm$^3$ for all NM20x; 1.75 g/cm$^3$ for all NM40x based on Kim et al. (2009).

$^b$ Standard deviation calculated over 3 repeats

$^c$ Measurement uncertainty as there was no repeat for this test

$^d$ Correspond to the LOD in mass dustiness index

Remarks on results including tables and figures

The Figure 8 below shows the respirable mass-based indices of all MN tested with the VS method. There is a wide variation in the indices of all MN. In both categories TiO2 and SiO2, the MN samples show behavior quite distinct. As observed for the SD method, the TiO2 samples show indices lower than the SiO2 samples. It is difficult to say something about the NTC because for two of them (NM400 and 401) the indexes were not significant, below the limits of detection (LODs). However, both NTC samples
NM402 and 403 shows indices quasi-equivalent, well above the LODs.

Figure 8: Respirable mass dustiness indices of MN tested with the VS method. The gray bars correspond to the LOD expressed in mass-based indices. Respirable mass dustiness indices are presented with their measurement uncertainty but not visible on the graph as below 1%.

Figure 9 shows the number (1/mg) and respirable mass (mg/kg) dustiness indices of MN tested with the VS method. The MN samples were classified according to their highest respirable mass index to the lowest. There is no correlation between the two presented indices, respirable mass or number. The ratio between the max and min values is similar for both number and respirable mass indices, it is ~100. For both NM400 and 401, the respirable mass collected on the cyclone filter was below the LOD of the weighing procedure. It is interesting to note that, for both NM400 and 401, while the results in respirable mass are below the detection limit, the values obtained in number of particles emitted are significant.
Figure 9: Number (1/mg) and respirable mass (mg/kg) dustiness indices of MN tested with the VS method.

In the following Figure 10 it can be observed the influence of test duration on the calculation of the number dustiness index (1/mg). The relationship between these two indices varies greatly from about a factor of 2 to over 50 (this is the case for the MN 203).

Figure 10: Number (1/mg) dustiness indices for all MN tested with the VS method as measured by CPC. Comparison of two test times T for calculation: 180 and 3600 s.

Figures 19, show the particle number size distributions for all TiO$_2$ samples obtained with the VS method and measured by the ELPI Classic (standard configuration). All distributions are presented as given by the
ELPI (aerodynamic equivalent diameter). For each MN sample, the particle number size distribution is shown for two values of the particle density (see above in chapter Error! Reference source not found.).

Figure 11: Particle number size distributions for TiO2 MN samples obtained with the VS method and measured by the ELPI Classic (standard configuration). All distributions are presented as given by the ELPI (aerodynamic equivalent diameter). Densities used were: 3.84 g/cm³ for NM100, 101, 102 and 4.26 g/cm³ for NM103, 104, 105 based on Teleki et al. (2008).

Overall remarks, attachments

Attached full study report

Attached document  D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1

Remarks  Dispersion protocol
Within this project two methods for characterizing the dustiness of nanomaterials in powder have been developed: Small Rotating Drum method and Vortex Shaker Method. The results of the present work suggest that:

- There are different dust generation rate time profiles. This difference in the dynamic of dust generation is reflected in the difference dustiness indices that are calculated.
- Both SD and VS methods gave reproducible results in terms of amount and size distribution of the generated particles for the NM samples in the project. All size distributions of as measured by the SD method were bi- or multimodal.
- Airborne particles generated during these tests are agglomerates/aggregates as shown by the few EM observations made on three selected NM. These results are in agreement with those of the existing literature.
- The comparison between the small drum and Vortex shaker shows that no significant correlation between the two can be found. Further evaluation of this method is needed in order to link it the standardized rotating drum method. Dustiness as quantified by particle number or by mass-based dustiness index had for both methods a larger range. These findings suggest a corresponding large difference in exposure potential. It is however difficult to say more to the extent the relationship between index dustiness and actual exposure is not known.

The comparison between the small drum and Vortex shaker shows that no significant correlation between the two can be found. Further evaluation of this method is needed in order to link it the standardized rotating drum method. Dustiness is not an intrinsic physical or chemical defined property of a powder, but its level depends on as well as characteristic properties of the powders and the activation energy in the simulated handling. Therefore different values may be obtained by different test methods (test apparatus, operation procedure, sampling and measurement strategy, etc.). It seems obvious that the absence of a harmonized approach concerning the measurement strategies and techniques, metrics and size ranges and the procedures of data analysis and reporting severely limits the comparison of these dustiness methods. Very little work has been done so far in this direction. That is why such a harmonized approach has been already integrated into various European research programs to be launched soon. One of them will be realized within the framework of the Mandate 461. Dustiness data obtained within this project can therefore contribute with information on the potential exposure risk level during powder handling (Schneider and Jensen, 2009). Size-distribution analysis of dustiness materials additionally may give information on the potential aggregate and agglomerate size of dust particles released from handling.

**Cross-reference to other study**


**4.32 Porosity**

**Endpoint study record: Specific surface area by BET by NANOGENOTOX**

**Administrative Data**

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72
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**Reference**

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**Data access**

other: owner:NANOGENOTOX

**Materials and methods**

**Methods**

BET

**Principles of method if other than guideline**

Surface area and porosity are important characteristics, in understanding the structure, formation and potential applications of different natural materials. For this reason it is important to determine and control them accurately. The most widely used technique for estimating surface area is the so-called BET method (Brünauer, Emmett and Teller, 1938) [5]. The concept of the theory is an extension of the Langmuir theory, which is a theory for monolayer molecular adsorption, to multilayer adsorption with the following hypotheses: (a) gas molecules physically adsorb on a solid in layers infinitely; (b) there is no interaction between each adsorption layer; and (c) the Langmuir theory can be applied to each layer.

**Details of methods and data evaluation**

BET analyzer operates by measuring the quantity of gas adsorbed onto or desorbed from a solid surface at some equilibrium vapor pressure. The data are obtained by admitting or removing a known quantity of adsorbate gas (Nitrogen) into or out of a sample cell containing the solid adsorbent maintained at a constant temperature below the critical temperature of the adsorbate (at temperature of liquid Nitrogen). As adsorption or desorption occurs the pressure in the sample cell changes until equilibrium is established. The quantity of gas adsorbed or desorbed at the equilibrium pressure is the difference between the amount of gas admitted or removed and the amount required to fill the space around the adsorbent (void space). Sample preparation: no special treatment needed. Measurements performed on powder. 0.1 g of the material placed it in the appropriate cell size (the volume of the sample may vary from sample to sample due to difference in density etc.). Details of the method and values of used parameters might be found in the attached file with full study report: Draft D4.4_specific surface area.

**Data gathering**

**Instruments**

High-speed surface area and pore size analyzer NOVA 4200e (Quantachrome) NOVA 4200e equipped with four preparation ports (vacuum or flow degassing) and four analysis ports. It provides single and multi-
point BET surface area with y-intercept, "C" constant, slope and correlation coefficient; up to 100 adsorption and 100 desorption isotherm points; B.J.H pore size distribution calculated from the adsorption or desorption isotherm; total pore volume and average pore radius.

**Reproducibility**

two measurements were performed

**Test materials**

**Test material equivalent to submission substance identity**

yes

**Reference Material/Nanomaterial and Sample identification number**

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**Test material identity**

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**State of test material**

other: fluffy powder

**Any other information on materials and methods incl. tables**

The results from the BET analyses conducted in the project was compared with manufacturers data. BET (manufacturer) (m2/g): > 250

**Results and discussions**

**Specific surface area**

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Remarks on results including tables and figures

Figure 4.10: Isotherms of nitrogen sorption experiments at 77K for the TiO₂ nanomaterials

Overall remarks, attachments

Attached full study report
Remarks: Dispersion protocol

Remarks: Data in the report and details protocol in annex

Applicant's summary and conclusion

Conclusions
see the endpoint: comparison between BET and SAXS

Cross-reference to other study
4.33 Pour density

4.34 Photocatalytic activity

4.35 Radical formation potential

4.36 Catalytic activity

5. ENVIRONMENTAL FATE AND PATHWAYS

5.1 Stability

5.1.1 Phototransformation in air

5.1.2 Hydrolysis

5.1.3 Phototransformation in water

5.1.4 Phototransformation in soil

5.1.5 Preliminary: Dispersion stability in water

*Endpoint study record: Dispersion stability in water (publication)*

**Administrative Data**

**Purpose flag**: ( ) robust study summary ( ) used for classification ( ) used for MSDS
**Study result type**: experimental result
**Reliability**: 2 (reliable with restrictions)

**Authors**: von der Kammer, F., Ottofülling, S., Hofmann, T. (2010),
**Title**: Assessment of the physico-chemical behavior of titanium dioxide nanoparticles in aquatic environments using multi-dimensional parameter testing. Environmental Pollution 12, 3472–3481
**Test material**: P25, Hombikat UV-100
**Source type**: publication (peer-review)
**Guideline**: no

**Subject**: Aggregation behaviour (colloidal stability) in aqueous solutions
**Test media + conditions**: Aqueous solutions with different pH (4-8), electrolyte (NaCl, CaCl2, Na2SO4), and NOM-concentrations (Suwannee river NOM) 60 datapoints per single matrix were tested. To prepare each matrix TiO2 stock suspensions were mixed with electrolyte/NOM solutions and titrated with NaOH and HCl solutions to the chosen pH-values.
**Study type**: laboratory test
**Test duration**: 15h
**Application method**: Stock suspensions of 50 mg/L TiO2 were prepared by suspending TiO2 particles in MilliQ water followed by ultrasonication for 30 min (2x60 W indicated power). 25 ml of these suspensions were then mixed with electrolyte and NOM solutions and Milli-Q water up to 50 mL total
volume using a Metrohm Titrando 836 auto titration system. The pH was adjusted using NaOH and HCl solutions.

**Endpoint:** Analysis of particle concentration, hydrodynamic diameter, and electrophoretic mobility in the supernatant.

**Chemical analysis, Material characterization:** The concentration of particles in the supernatant after 15h settling time was determined by measuring the nephelometric turbidity (Hach 2100N IS Turbidimeter, LED light source λ=870nm) Particle size and zeta potential via DLS and electrophoretic mobility (Malvern Zetasizer Nano ZS).

**GLP:** no

**Validity criteria according to the guideline fulfilled:** --- (no guideline study)

**Test concentrations:** 25 mg/L final concentration

**Suitability of applied methods:** no

**obvious limitations Deviations from standard procedure:** no (no standard procedure)

**Results:** The behaviour of the tested particles in the different matrices follows in general the expectations derived from classical DLVO-theory for metal oxides with variable surface charge. The particles are stabilized in the presence of NOM due to charge reversal or increasing surface charge because of NOM-adsorption to the surface. Both particles behave similar in NaCl and CaCl2 matrices in general. P25 showed an anomalous charge reversal effect resulting in increased particle stability with increased NaCl concentration. Reactions with CaCl2 were similar for both materials with particle stabilization at low and high electrolyte concentration indicating a specific interaction of Ca2+ and the TiO2. High concentration of a divalent anion like SO42- destabilized both particles. Na2SO4 addition to the matrix leads to elevated zeta potentials at pH>IEP compared to NaCl solutions. Increased particle stability at low Na2SO4 concentrations and zeta potentials indicate that the classical correlation between low surface charge and low particle stability does not always exist in the presence of multivalent anions like sulphate.

**Information concerning test and procedure:**

**Is the information comprehensively and sufficiently:** yes

**Remark:** --

**Reliability** – adapted from Klimisch et al (1997): 2c

**Conclusions**

**Endpoint study record: Dispersion stability in water, University of Vienna (1)**

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Reliability** 2 (reliable with restrictions)

**Authors:** von der Kammer,F., Hofmann, T. (2012),

**Title** Testing the OECD selected alternative nano-TiO2 materials for dispersion stability, environmental behaviour and fate.

**Source** :Project report, University of Vienna, Department of Environmental Geosciences

**Test material:** P25, Hombikat UV100, UV-Titan M212, UV-Titan M262, PC-105, Tiona AT-1

**Source type:** Project report

**Guideline:** ---

**Subject:** Dispersion stability and environmental fate of P25 compared to alternative materials

**Test media + conditions:** In the proposed test system a stable dispersion of the to-be-tested particles is separated into 300-450 subsamples and each is then subjected to a different hydrochemical condition. This results in a three dimensional matrix of dispersion stability over pH and ion concentration. By
applying different salts as NaCl, CaCl2 etc. a set of matrices is obtained, the results become multi-dimensional.

**Study type:** laboratory test  
**Test duration:** 12-15h

**Application method:** Weighing of 50/100/250mg in 1L MQ water, adjusting pH to 7-7.5 with either 1 mol/L HCl or NaOH, 30 sec. ultrasonic bath treatment, followed by a wetting time of 24 hours. Using ultrasonic bath to disperse the particles (120W output, constant, 60 min.), adjusting pH to 7-7.5 as before. In the described test a stable dispersion of TiO2 in ultrapure water is subjected to a change in water chemistry and the phase separation (aggregation and settling of the particles) is measured once after a given time period.

**Endpoint:** TiO2 nanoparticle concentration, particle size and electrophoretic mobility.

**Chemical analysis, Material characterization:** The concentration of TiO2 in the supernatant was determined by measuring the nephelometric turbidity (Hach 2100N IS Turbidimeter, LED light source = 870). Particle size by DLS (Zetasizer ZS).

**GLP:** no

**Validity criteria according to the guideline fulfilled:** --- (no guideline study)

**Test concentrations:** 25 mg/L TiO2 concentration

**Suitability of applied methods:** for stable suspensions only, water dispersible Nanomaterial, only

**Deviations from standard procedure:** no (no standard procedure)

**Results:** It could be shown that the synthetic test results relate well to the reactions observed in a real setting using various natural samples and test media (Ottofuelling et al 2011). The synthetic test results however cover a much broader range of conditions than single real world testing could offer. The four materials could be clearly distinguished from each other and are expected to show different behaviour in the environment. One material (Hombikat UV100) behaves unlike a typical bare TiO2 material. The test system was able to clearly show similarities and differences between the different materials. The developed multi-dimensional test system enables the direct assessment of dispersion stability for the environmental fate testing of engineered nanoparticles. It also serves as an experimental basis to investigate the general dispersion behaviour of nanoparticles and may be applied to compare the effects of different surface coatings and functionalizations.

**Information concerning test and procedure:**

Is the information comprehensively and sufficiently: yes

**Remark:** ---

**Reliability – adapted from Klimisch et al (1997):** 1d

**Conclusions**


---

5.2 Biodegradation

5.2.1 Biodegradation in water: screening tests

5.2.2 Biodegradation in water and sediment: simulation tests

**Endpoint study record: Waste water treatment simulation. (publication)**

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Reliability** 2 (reliable with restrictions)
Data source
Reference
Reference type publication
Author Kiser, M.A., Westerhoff, P., Benn, T., Wang, Y., Perez-Rivery, J., and Hristov, K.
Year 2009
Title Titanium Nanomaterial Removal and Release from Wastewater Treatment Plants
Bibliographic source Environmental Science & Technology 43 (17), 6757–6763

Applicant's summary and conclusion

Executive summary
Test material: Hombikat UV 100 Source type: publication (peer-review) Guideline: --- Subject: Adsorption of Titanium Dioxide with Wastewater Biomass Test media + conditions: Batch Adsorption Isotherm Experiment: A TiO2 test solution (1 mM NaHCO3; pH 7.2) was mixed continuously and equilibrated for 10 h. A wastewater bacteria biomass stock solution was prepared by rinsing the activated sludge three times with a 1 mM NaHCO3 solution and then resuspending the sludge in the rinsing solution. A series of TiO2 test solution were spiked with varying amounts of biomass stock solution and then agitated for 3 h. After agitation, the biomass was allowed to settle by gravity (about 30 min), and then 20 mL of supernatant was collected from each sample and analysed for Ti after digestion. WWTP-simulation: Sequencing batch reactors (SBRs) were used to represent the full-scale WWTP operations of aeration and settling. SBRs were constructed using 2 L reactors supplied with compressed air and mechanical mixing units. The SBR contained heterotrophic bacteria acclimated to a feed solution (668 mg/L C5H8NO4Na, 44 mg/L KH2PO4, 90 mg/L MgSO4 •7H2O, 14 mg/L CaCl2 •2H2O, 10 mg/L yeast extract, and 0.3 mL/L nutrient solution) and was operated to maintain a total volume of 1.6 L, a hydraulic residence time (HRT) of 10 h, and a solids retention time (SRT) of 6 days, which is typical of aerobic WWTPs. The SBR cycle involved 8 h of aeration plus mixing, followed by 2 h of settling time. Removed solutions were analysed for concentrations of Ti and suspended solids and were replaced with the same volumes of feed solution. Study type: laboratory test Test duration: Batch adsorption: 3h, WWTP-simulation: 6 days Application method: A nanoscale TiO2 suspension was prepared by adding TiO2 to ultrapure water, sonicaing for 1 h (200 W/L), and centrifuging at 1000 g for 30 min. Following centrifugation, the supernatant containing suspended TiO2 was removed and used as stock solution. For Batch experiments a test solution of 2 mg Ti/L was prepared from the stock solution. WWTP-simulations containing a feed solution only (no biomass) or feed solution plus biomass were supplied with 2.9 ± 0.3 mg Ti/L during each liquid exchange. Endpoint: Ti concentration in supernatant and solid sample material; partitioning behavior Chemical analysis, Material characterization: The supernatant was analysed for Ti (ICP-OES) after digestion with aqua regia. Electron microscopy (SEM/EDX) after partial digestion with 30% H2O2. GLP: no Validity criteria according to the guideline fulfilled: --- (no guideline study) Test concentrations: 2-3 mg/L final Ti concentration Suitability of applied methods: no obvious limitations Deviations from standard procedure: no (no standard procedure) Results: As increasing dosages of biomass (in solutions of the same ionic strength) were added to the samples, more TiO2 was removed from the supernatant; A comparison of the Ti concentrations in the control and the sample containing 2250 mg/L TSS shows that approximately 85% of the Ti was removed from suspension. Removal data were fit by a Freundlich Isotherm. Results suggests that nonlinear partition models may be required to describe nanomaterial biosorption removal in WWTPs, which differs from the linear partition models often used for most organic pollutants. Overall, only 12% of the Ti passed through the WWTP simulation in the supernatant, while 88% was associated with the biosolid fraction. These experimental results reveal the high affinity of TiO2 for biomass. At more complex wastewater matrix a lower degree of Ti was removed (69%). Wastewater contains surfactants and natural organic matter, which have been shown to hinder the removal of some nanoparticles from water. Though not completely equivalent, both systems lead to the conclusion that while significant fractions of Ti will associate with biomass and be present in finished biosolids, a portion of the Ti will also be present


in WWTP effluents that enter the aquatic environment. Information concerning test and procedure: Is the information comprehensively and sufficiently: yes  Remark: ---  Reliability – adapted from Klimisch et al (1997): 2e

*Endpoint study record: Waste water treatment simulation. (Publication)*

**Administrative Data**

- **Purpose flag**  ( ) robust study summary ( ) used for classification ( ) used for MSDS
- **Study result type**  experimental result
- **Reliability**  2 (reliable with restrictions)

**Data source**

- **Reference type**  study report
- **Author**  Wang, Y., Westerhoff, P. and Hristovski, K.
- **Year**  2012
- **Title**  Fate and biological effects of silver, titanium dioxide, and C60 (fullerene) nanomaterials during simulated wastewater treatment processes

**Applicant's summary and conclusion**

**Executive summary**

Test material: Hombikat UV 100  Source type: publication (peer-review) Guideline: ---  Subject: Adsorption of Titanium Dioxide in WWTP simulation  Test media + conditions: Batch experiments in reactors with a volume of 1.6 L. The reactors were seeded with bacteria culture (return activated sludge) from WWTP. The reactors were supplied with a synthetic feed solution comprised of salts, trace nutrients, buffer and monosodium glutamate (C5H8NO4Na) as a carbon and nitrogen source. This feed solution had a conductivity of 0.5 mS, COD of 780 mg/L, and total dissolved nitrogen (TDN) of 150 mg N/L. the hydraulic residence time (HRT) was 8 h plus settling. The sludge retention time (SRT) was managed in most test at 6.4 day. Control reactors were operated with (1) the feed solution with NM but no biomass and (2) the feed solution with no NM. Study type: laboratory test  Test duration: 6 days  Application method: The stock suspension was prepared by suspending 1 g of TiO2 into 1 L of ultrapure water and sonicating it with an ultrasonic probe (5T Standard Probe, Model 2000U, Ultrasonic Power Cooperation, Freeport, IL, USA) for 2 h at 200 W/L.

**Results:**

When the n-TiO2 was added into the feed solution, the nanoparticles rapidly aggregated and formed large particles (>1 mm). In an attempt to stabilize n-TiO2 in the control reactors, 5 mg DOC/L of NOM was fed with n-TiO2. Most wastewater effluents contain 4–15 mg/L of DOC. However, NOM had minimal effect on n-TiO2 removal, presumably because divalent cations (Mg2+ and Ca2+) present in the feed solution still complexed with the NOM coatings on the NM and facilitated their aggregation. The removal of titanium increased from 65% in the absence of biomass to 97±1% with biomass present.
Clusters of aggregated n-TiO2 with a size of several hundred nanometers were present in the biosolids but most of the titanium dioxide remained in nanoparticle form. The data initially showed a gradual increase in the total Ti concentration in the biosolids, which began to plateau after 15–18 days, approximately three times the SRT value of 6.4 days. After reaching the plateau the biosolids contained approximately 8 mgTi/gSS Overall, the data collected indicate that biological wastewater treatment plants operated using suspended biomass (e.g., activated sludge) have the potential to remove engineered nanomaterials from wastewaters by interaction with biomass in systems operated with TSS similar to that of full-scale WWTPs. Information concerning test and procedure: Is the information comprehensively and sufficiently: yes  Remark: --- Reliability – adapted from Klimisch et al (1997): 2e

5.2.3 Biodegradation in soil

5.3 Bioaccumulation

5.3.1 Bioaccumulation: aquatic / sediment

5.4 Transport and distribution

5.4.1 Adsorption / desorption

5.4.2 Henry's Law constant

5.4.3 Distribution modelling

5.4.4 Other distribution data

Endpoint study record: Transportation behaviour in model plume (publication)

Administrative Data

Purpose flag  ( ) robust study summary ( ) used for classification ( ) used for MSDS

Data source

Reference

Reference type publication
Author Battin,T.J., Kammer,F., Weilhartner,A., Ottofuelling,S., and Hofmann,T.
Year 2009
Title Nanostructured TiO2: Transport Behavior and Effects on Aquatic Microbial Communities under Environmental Conditions
Bibliographic source Environmental Science & Technology 43, 8098-8104

Applicant's summary and conclusion

Executive summary

Test material: P25, Hombikut UV-100 Source type: publication (peer-review) Guideline: --- Subject: Transportation behaviour in model plume Test media + conditions: Natural surface water (chemically characterised) amended with nutrients and glucose. Microcosms set up with plume and water recirculation, triplicate microcosms with plume for each of the test materials with biofilm, duplicate microcosm with plume without biofilm, prior to each test microcosm without plume (system), 24 d biofilm growth on glass
slides under simulated light, afterwards recirculation (plume water depth 15 mm, volume 1400 ml, final concentration of TiO2 5.3 mg/L), recirculation of TiO2 for 48 h under simulated light, online turbidity determination, frequent particle size determination. Study type: laboratory test  Test duration: 48 h Application method: as suspension in filtered (0.45 µm; cellulose acetate membrane) surface water (Lunzer Unteree, Austria) Treatment: Ultrasonication (probe sonication) of suspension of TiO2-samples in water (30s, 25W at 10% energy input) Application: injection of probes into plume of microcosms. Endpoint: TiO2-concentration and particle size in water column during recirculation period Calculation of travel distance Total mass of NM deposited after 3 h Chemical analysis, Material characterization: Particle concentration by turbidity measurement, size by DLS, TEM control measurements. GLP: no Validity criteria according to the guideline fulfilled: --- (no guideline study) Test concentrations: 5.3 mg/L final concentration Suitability of applied methods: no obvious limitations  Deviations from standard procedure: no (no standard procedure) Results: In the control flume without biofilm, P25 and Hombikat UV-100 nanoparticles travelled on average 10 km and 12 km downstream, respectively, before 99.9% of their initial mass concentration was removed from the water. Biofilms reduced the travel length on average 2.3 times for Hombikat UV-100 and 2.7 times for P25. The deposition induced accumulation in the biofilms. Very artificial system only. Transferability of travel length in natural streams is questionable. Information concerning test and procedure: Is the information comprehensively and sufficiently: yes  Remark: --- Reliability – adapted from Klimisch et al (1997): 2e

5.6 Other relevant information

6. ECOTOXICOLOGICAL INFORMATION

6.1 Aquatic toxicity

6.1.1 Short-term toxicity to fish

*Endpoint study record: Short-term toxicity to fish. by RWTH-Aachen University, Germany*

**Administrative Data**

- **Purpose flag**  key study ( ) robust study summary ( ) used for classification ( ) used for MSDS
- **Study result type**  experimental result
- **Study period**  July 2013
- **Reliability**  1 (reliable without restriction)

**Data source**

**Reference**

- **Reference type**  study report
- **Author**  Anne Wyrwoll
- **Testing laboratory**  Institute for Environmental Research, RWTH-Aachen University, Germany
- **Report no.**
- **Owner company**  Federal Environment Agency Germany, Institute for Environmental Research, RWTH-Aachen University, Germany
Data access
data submitter is data owner

Cross-reference to same study
The nanomaterial NM 102 and the non nano scale titanium dioxide material NM 100 were tested parallel to NM 101 in the fish embryo toxicity test.

Materials and methods

Test guideline

Qualifier according to
Guideline other guideline: OECD 236
Deviations yes

Principles of method if other than guideline
Adult fish were maintained with a light dark rhythm of 14:10 hours. When the light was turned on in the morning, eggs were produced via mass spawning of a group of fish consisting of a gender ratio of 1:2 female and male fish. Directly after spawning fertilized eggs which were within the 8-cell and 64-cell stages, undergoing normal cleavage and showing no injuries of the chorion were selected by using a binocular microscope. To prevent loss of the titanium dioxide material by sedimentation, selected eggs were transferred directly into the test vessels (24-well plastic plate) and not into pre-incubation vessels. Five eggs were transferred within 1 ml 10% higher concentrated reconstituted water (HCRW, ISO 7346) to a test medium volume of 9.0 ml. In the end the test medium consisted of 80% HCRW and 10% deionized water or TiO2 suspension (stock or working suspension) or a mixture of both, depending on the treatment group. Stock suspensions were prepared as described in the SOP ‘Preparation of a NM 101 suspension’ prior to testing and were diluted to the working suspension with deionized water (100 mg/L). After test vessels were covered with an air permeable membrane they were placed in a 26°C tempered incubator under dark conditions. Embryos were exposed for 72 h without replacement of the test medium. Sub-lethal and teratogenic effects were recorded every 24 h. NM 101 was tested at three different concentrations (1, 10 and 100 mg/L) with 10 eggs per concentration. 40 control eggs were placed into test medium consisting of HCRW and deionized water only. Additionally, 20 eggs were exposed to the positive control 3,4-dichloroaniline (3.7 mg/L). In a second independent test 10 eggs were exposed to the highest NM 101 concentration (100 mg/L) under the same test conditions. In total 20 eggs were exposed in two individual experiments to the highest test concentration. This meets the requirements of the OECD guideline for a limit test.

GLP compliance
no in the style of GLP

Test materials

Analytical monitoring
No

Test organisms

Test organisms (species)
Danio rerio
**Details on test organisms**
Adult fish (Danio rerio) were derived from the division Applied Ecology of the institute for Molecular Biology and Applied Ecology in Schmallenberg, Germany. They were maintained in dechlorinated tap water at 26°C with a light dark regime of 14:10 hours.

**Study design**

**Test type**
static

**Water media type**
freshwater

**Limit test**
yes

**Total exposure duration**
72 h Remarks

**Test conditions**

*Test* temperature
26°C

**Reference substance (positive control)**
yes 3,4-dichloroaniline

**Results and discussions**

**Effect concentrations**

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<th>72 h</th>
</tr>
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<td>Effect conc.</td>
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<td>Basis for effect</td>
<td>mortality</td>
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<td>Remarks (e.g. 95% CL)</td>
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<th>Duration</th>
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<td>Conc. based on</td>
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<td>Basis for effect</td>
<td>mortality</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
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</tr>
</tbody>
</table>

**Details on results**
NM 101 did not show any effect on the mortality or hatching rate of D. rerio within an exposure period of 72 h.
Results with reference substance (positive control)
80% mortality was observed for the positive controls after an exposure period of 72 h. This result seems to be in line with the criteria given in the guideline (4 mg/L exposure results in a minimum mortality of 30% after an exposure period of 96 h).

Overall remarks, attachments
Attached background material
Attached document 2: SOP Preparation of a NM 101 suspension.pdf:
ENV/JM/MONO(2015)17/PART3/ANN1

Applicant's summary and conclusion
Validity criteria fulfilled
yes

Conclusions
NM 101 had no effect on the mortality of D. rerio embryos up to a concentration of 100 mg/L under the conditions tested. As nano-titanium dioxide is known to be photoactivated by illumination with solar radiation, it stays unclear whether NM 101 would induce toxic effects on D. rerio during a parallel exposure to solar radiation. This was not examined in the present study, but should be considered in further studies.

Endpoint study record: zebrafish by INIA.004

Administrative Data
Purpose flag  ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source
Data access
other: performed and provided by INIA, Spain

Materials and methods
Principles of method if other than guideline
Toxicity tests were conducted in accordance with the OECD TG 212 Fish, short-term toxicity test on embryo and sac fry stages

Test materials
Details on test material
NM 101, Titanium Dioxide 91.7% Modification Anatase BET surface area >250 m²/g.

Details on test solutions
The following exposure concentrations of NM: 10, 100 and 1000 mg/l were prepared as for D. magna tests with the following modifications. Stock solution of 1000 mg/l of NM was prepared in embryo water 90
μg/ml Instant Ocean (Aquarium Systems, Sarrebourg, France), 0.58 mM CaSO₄, 2H₂O, dissolved in reverse-osmosis purified water 24 h before applying to zebrafish embryos. Likewise for D. magna stocks and test solutions of NM were sonicated for 2 min at 50% amplitude and then stirred for 24 h prior to tests.

Test organisms

Test organisms (species)
Danio rerio

Details on test organisms
embryos and larvae 2 h after spawning

Study design

Test type
static

Total exposure duration
8 d

Remarks

Test conditions

Test temperature
28.5 °C

Details on test conditions
Toxicity tests were conducted in accordance with the OECD TG 212 Fish, short-term toxicity test on embryo and sac fry stages as follows: Zebrafish (Danio rerio) embryos and larvae were obtained by natural mating and raised at 28.5 °C on a 12L:12D photoperiod. Within 2 h after spawning, 10 embryos were transferred to 6-well plates containing 5 mL of test medium (two replicated per treatment and NM). Embryos were exposed for 8 days in a dark incubator at 28.5 °C without food. Hatching status, survival and any obvious morphological abnormalities were noted daily. Body length and morphological abnormalities were monitored in fixed 8 dpf larvae as follows: larvae were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, followed by several washes in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 0.02 M PO₄) and gradually transferred to 90% glycerol. Embryos and larvae were examined with a Nikon SMZ 1500 stereomicroscope to observe the phenotype. Differential interference contrast images or videos were obtained using a Nikon Eclipse E1000 (Nikon, Champigny sur Marne, France) microscope fitted with Nomarski optics. Images were acquired with a Nikon DXM1200 camera and LUCIA G version 4.81 software. Total body length (anterior-most part of the snout to posterior-most point of the tail) were measured on the left side of each fish. All images for morphometric analysis had a constant number of pixels per inch. Total body lengths were determined by drawing a line to obtain the length in pixels.

Results and discussions

Details on results
At the end of tests no effects on hatchings and mortality was observed in any of the treatments performed (Table 1). All larvae had the same normal developmental larvae stage at 8 dpf (Fig 2). Body length measurements denoted significant differences staged at day 8. Significant differences on body length relative to controls were only observed in 8 dpf larvae exposed at 1000 mg/l of NM 105.
Results with reference substance (positive control)
control group receiving only embryo medium

Reported statistics and error estimates
Body length measurements were compared using one way ANOVA followed by post hoc Dunnett’s test. No effect concentration values (NOEC) were obtained from low effect values (LOEC) relative to control treatments using one way ANOVAs followed by one side Dunnett’s post hoc set with P< 0.05. Stats were conducted using the IBM SPSS 19.0 software.

Any other information on results incl. tables
10 embryos exposed to 10, 100 and 1000 mg/l of each TiO2 NM, two replicated per treatment and NM Method of calculating mean measured concentrations: arithmetic mean

*Acute Toxicity to Fish

Departmento de Medio Ambiente (Department of Environment)
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Madrid, Spain

Date: 21.06.12

Table 1. Hatching and survival of exposed embryos to the studied NM after 8 days of exposure.

<table>
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<th>TREATMENT</th>
<th>Exp(mg/l)</th>
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<th>% survival</th>
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Treatment P-25 EVONIK -1000

Treatment NM 101-1000

Treatment NM 103-1000

Treatment NM 104-1000

Treatment NM 105-1000
Fig 2. Images of 8 dpf larvae in the different treatments at 1000 mg/l.

Fig 3. Mean ± SE body length of 8 dpf larvae exposed to the studied NM at 10, 100 and 1000 mg/L concentrations.

Applicant's summary and conclusion

Conclusions
LOEC for growth were then set to 1000 mg/l for NM 105. Accordingly NOEC could only be obtained for NM 105 and were 100 mg/l

Overall remarks, attachments
Attached background material
Zebrafish_acute final tables and pictures.doc

6.1.2 Long-term toxicity to fish

6.1.3 Short-term toxicity to aquatic invertebrates

Endpoint study record: Short-term toxicity to aquatic invertebrates by RWTH-Aachen University

Administrative Data

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<th>key study ( ) robust study summary ( ) used for classification ( ) used for MSDS</th>
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Data source

Reference

Reference type  study report
Author  Anne Wyrwoll
Year
Materials and methods

Testing laboratory  RWTH Aachen
Owner company  UBA, RWTH Aachen

Data access
data submitter is data owner

Materials and methods

Test guideline
Qualifier  according to
Guideline  OECD Guideline 202 (Daphnia sp. Acute Immobilisation Test)
Deviations

Principles of method if other than guideline
Acute toxicity tests were performed with < 24 h old neonates of Daphnia magna according to OECD guideline 202 (48 h exposure duration). Two parallel test series were run with either laboratory light (LL) or simulated solar radiation (SSR) under a 16 h light/8 h dark regime. Each test series consisted of five treatment groups with different concentrations and one control. Each treatment group consisted of four replicates containing each five neonates. Every test stock suspension (1 g/L) was prepared as explained in the standard operating procedure (SOP) "Preparation of a NM 101-suspension". Working suspensions (100 mg/L) were diluted from the stock suspension with deionized water. Afterwards, the desired test concentrations were obtained by diluting either the stock dispersions or the working dispersion with test medium. 10 fold diluted ISO water (undiluted ISO water was prepared as recommended in the OECD guideline 202) was used as test media for the immobilization tests. Light sources: Laboratory light: A normal fluorescent tube was used for testing under laboratory light. As described before, a 16 h light/8 h dark regime was used. Simulated solar radiation: A metal vapor lamp emitting visible radiation comparable to sunlight (280-800 nm) was used (Bright Sun UV Desert, 70 W, Lucky Reptile, Waldkirch, Germany) as light source for the testing with SSR. The distance between the lamp and the test vessels was 70 cm. The manufacturer states that the irradiance of the UVA and UVB radiation of the lamp (3.2 mW/cm² and 50 µW/cm²) is comparable to the corresponding irradiance of solar radiation (5 mW/cm² and 260 µW/cm²) at a midsummer day in Germany. The irradiance of the UVA and UVB radiation as given by the manufacturer is comparable with the irradiance of natural sunlight in the troposphere. As described before, a 16 h light/8 h dark regime was used.

GLP compliance
no in the style of GLP

Test organisms
Test organisms (species)
Daphnia magna
Details on test organisms
< 24 h old neonates of Daphnia magna according to OECD guideline 202
Study design

Test type
static

Water media type
freshwater

Limit test
no

Total exposure duration
48 h

Remarks

Test conditions

Test temperature
19-20°C

pH
6.58-7.65 The variation of the pH in the test medium between the test initiation and termination in each test was not greater than 1.5 units.

Dissolved oxygen
7.0-8.7 mg/L

Details on test conditions
Tests were performed with simulated solar radiation and with laboratory light. Furthermore 10 fold diluted ISO medium was used instead of ISO medium as recommended in the OECD guideline 202. For details please see remarks in the section "Principal of method if other than guideline".

Results and discussions

Effect concentrations

Duration
48 h

Endpoint
EC50

Effect conc.
79.52 mg/L

Nominal/Measured
nominal

Conc. based on
test Basis mobility ; Results of the laboratory light test.
mat. for effect

Remarks (e.g. 95% CL)
95%-CL 62.64/112.71 mg/L (lower/upper)

Duration
48 h

Endpoint
LOEC

Effect conc.
33.3 mg/L

Nominal/Measured
nominal
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<td>Duration</td>
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<td>Duration</td>
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<td>Conc. based on</td>
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**Reported statistics and error estimates**

Data were statistically analyzed with ToxRat® Professional (version 2.10, ToxRat solutions GmbH). Concentration response functions were fitted to the data using probit analysis. The median effective
concentration (EC50 was calculated from this function. Significant difference to the control (*P<0.05) were determined using Fisher’s Exact Binominal Test with Bonferroni Correction to derive the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC).

**Overall remarks, attachments**

**Attached background material**

Attached document 2: SOP Preparation of a NM 101 suspension.pdf:
ENV/JM/MONO(2015)17/PART3/ANN1

**Applicant's summary and conclusion**

**Conclusions**
The results of the present study show that toxicity of NM 101 to D. magna was promoted by environmental realistic levels of SSR. It is suggested that SSR induced toxicity is a consequence of SSR induced reactive oxygen species (ROS) production through the TiO2 material. NM 101 also showed a low toxicity under laboratory light exposure, indicating that either the material itself is toxic or that ROS production through NM 101 is already induced at wavelengths included in laboratory light. This study emphasizes the need for testing nano-TiO2 under environmental realistic levels of SSR in ecotoxicity assays because SSR induced ROS production seems to be a main mechanism of TiO2 toxicity.

**Endpoint study record: Daphnia magna by INIA**

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Data source**

other: performed and provided by INIA, Spain

**Materials and methods**

**Test guideline**

**Qualifier** according to

**Guideline** OECD Guideline 202 (Daphnia sp. Acute Immobilisation Test)

**Deviations**

**Test materials**

**Details on test material**

NM 101, Titanium Dioxide 91.7% Modification Anatase BET surface area >250 m²/g

**Details on sampling**

Stock solution: 1.5 g/l of TiO2 NM were prepared as follows: 0.15 g of TiO2 was diluted in 100 mL of ASTM hard water (ASTM 1980), sonicated for 2 min at 50% amplitude and stirred for 24 h prior to tests. Test concentrations of 100, 200, 400, 800, 1000, 1500 mg/l plus a control with no added NM were prepared from the stocks and sonicated for 30 min in a water bath.
Details on analytical methods
The percentage of immobile individuals was used as endpoint, transformed into probits and used to
determine LC50s (Finey 1971).

Test organisms

Test organisms (species)
Daphnia magna

Details on test organisms
Neonates < 24 day old

Study design
Total exposure duration
48 h Remarks

Test conditions
Test temperature
20 ± 0.5 °C

pH
7.8 to 8.1

Dissolved oxygen
95-100% of oxygen saturation levels that varies between 9 and 10 mg O2/L

Salinity
; 350-410 uS/cm conductivity

Nominal and measured concentrations
Test concentrations of 100, 200, 400, 800, 1000, 1500 mg/l plus a control with no added NM

Details on test conditions
Test were initiated with groups of ten neonates < 24 day old distributed in 20 ml of test medium in 30 mL
borosilicate glass jars. Three replicates per concentration were conducted and at least two trials per
nanomaterial. Tests were conducted in a constant temperature room (20 ± 0.5 °C) without food and lasted
48 h. Oxygen levels, conductivity and pH were monitored at the start and end of acute tests. Dissolved
oxygen concentration (DO) was measured using a polarographic oxygen electrode coupled to a
CyberScan DO 300/3001 EUTECH model meter (Lab Process Distributions, Alella, Barcelona, Spain).
pH and conductivity were measured using an epoxy-body combination electrode, coupled to a Crison
micro pH 2001 and conductivity meters calibrated with standard pH and conductivity buffer solutions
(Sigma, Madrid, Spain). Temperature was recorded in continuum with a temperature probe developed and
calibrated at IDAEA, CSIC (Barcelona)

Any other information on materials and methods incl. tables
without food 14:10 h light: dark, 400 lux
Results and discussions
Details on results
Mortality was negligible < 10% in all tested exposure levels, thus it was not possible to estimate any LC50 < 1.5 g/l.

6.1.4 Long-term toxicity to aquatic invertebrates

Endpoint study record: Daphnia magna by INIA

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source
Data access
other: performed and provided by INIA, Spain

Materials and methods
Test guideline
Qualifier
Guideline OECD Guideline 211 (Daphnia magna Reproduction Test)
Deviations

Test materials
Details on test material
NM 101, Titanium Dioxide 91.7%, Modification Anatase, BET surface area >250 m²/g

Details on sampling
1.5 g/l of TiO2 NM stock solutions were prepared as follows: 0.15 g of TiO2 was diluted in 100 mL of ASTM hard water (ASTM 1999), sonicated for 2 min at 50% amplitude and stirred for 24 h prior to tests. Stocks of bulk TiO2 were prepared similarly. Stocks were kept at 4 °C for one week and prior to tests were sonicated again and stirred for at least 2 h.

Test organisms
Test organisms (species)
Daphnia magna

Study design
Test type
semi-static

Total exposure duration
21 d Remarks
Test conditions

**Test temperature**
20 ± 0.5 °C

**pH**
7.8 to 8.1

**Dissolved oxygen**
95-100% of oxygen saturation levels

**Salinity**
350-410 uS/cm conductivity

**Nominal and measured concentrations**
Test solutions of 0, 1, 3, 10 mg/L plus a control with no added NM

**Details on test conditions**
Test solutions of 0, 1, 3, 10 mg/L plus a control with no added NM were prepared by adding appropriate amounts of a concentrated stock solution to ASTM hard water, and the solution sonicated for 30 min in a water bath before adding algae and the test individuals. Individuals were maintained in 100 mL of ASTM hard water, in 120 ml screw top glass jars, with the addition of a standard organic extract (Baird et al., 1989). In all treatments animals were fed with Chlorella vulgaris Beijerinck (5 x 105 cells mL⁻¹) and maintained at 20 °C and with a photoperiod of 16 h: 8 h light: dark. Exposure solutions with algae were changed every other day. Test start with <24 h old neonates and finished after 21 days. During the tests deaths and offspring production was monitored daily and at the end of test the size of live individuals was measured. Due to the elevated number of material tested two different chronic test were performed between November 2010 (Exp.1) and March 2011(Exp.2). In each one four test substances plus a control treatment were tested and compared using separated ANOVA tests. Oxygen levels, conductivity and pH were monitored in freshly and old tests medium for chronic tests using. Dissolved oxygen concentration (DO) was measured using a polarographic oxygen electrode coupled to a CyberScan DO 300/3001 EUTECH model meter (Lab Process Distributions, Alella, Barcelona, Spain). pH and conductivity were measured using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 and conductivity meters calibrated with standard pH and conductivity buffer solutions (Sigma, Madrid, Spain).

Results and discussions

**Effect concentrations**

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<tbody>
<tr>
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<td>Duration</td>
<td>21 d</td>
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<td>1 mg/L</td>
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<tr>
<td>Nominal/Measured</td>
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</tbody>
</table>
**Details on Results**
Mortality was negligible during tests. Only in one treatment (bulk TiO2 at 10 mg/L 2 out of 10 individuals died). In any of the test performed there were significant (P<0.05) differences in the length of the individuals at the end of tests relative to control treatments (Fig 1), which indicate that NM of TiO2 did not affect somatic growth. Population growth rate significant (P<0.05) effects were observed at 3 mg/L.

**Reported Statistics and Error Estimates**
No effect concentration values (NOEC) were obtained from low effect values (LOEC) relative to control treatments using one way ANOVAS followed by one side Dunnett’s post hoc set with P< 0.05. The endpoints compared were body length and total offspring production at the end of tests (21 days) and population growth rates (r). Stats were conducted using the IBM SPSS 19.0 software. Body length measurements were performed from the head to the base of the spine with the aid of an ImageJ software (http://rsb.info.nih.gov/ij/) using a Nikon stereoscope microscope (SMZ 150, Nikon, Barcelona, Spain). The intrinsic rate of increase (r) was then computed iteratively from the Lotka equation (eq 1, see attachment) using the measured age specific survival and fecundity rates: where lx is the proportion of the females surviving to age x (days) and mx is the number of juveniles produced per surviving female between the ages x and x+1. The age at birth was set to 0. The 95% confidence intervals were estimated by the Jackknife method according to Meyer et al. (1986). LOEC indicated previously correspond to statistical differences for P<0.05.
B. Chronic Toxicity to Aquatic Invertebrates

Departamento de Medio Ambiente (Department of Environment)
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Madrid, Spain

Date: 21.06.12

\[ \sum_{x=0}^{\infty} e^{-rx} l_x \quad m_x = 1 \]  

(equation 1)

where \( l_x \) is the proportion of the females surviving to age \( x \) (days) and \( m_x \) is the number of juveniles produced per surviving female between the ages \( x \) and \( x+1 \). The age at birth was set to 0. The 95% confidence intervals were estimated by the Jackknife method according to Meyer et al. (1986).

Cite statistical methods used and appropriate reference(s).
Fig 1. Chronic responses of D. magna individuals exposed to the studied compounds (Mean SE, N=9-10). Asterisk indicate those groups significant (P<0.05) different from controls following ANOVA and one side Dunnet’s test.

**Overall remarks, attachments**

**Attached background material**
Daphnia chronic diagramme.doc

**Endpoint study record: Hyalella azteca by Institute of Water Science by Wilfrid Laurier University**

**Administrative Data**

**Purpose flag**  ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type**  experimental result

**Data source**

**Data access**
other: performed and provided by Wilfrid Laurier University
Cross-reference to same study
Malhi, Gurki and McGeer James. 2012. Dept of Biology, Wilfrid Laurier University Manuscript in preparations

Materials and methods

Test guideline
Qualifier according to
Guideline other guideline: EPS/11RM/33
Deviations

Test materials

Details on test material
from OECD Batch Ti from AAS standards (Sigma-Aldrich Inc. St. Louis, MO), which is called ‘dissolved Ti’

Details on analytical methods
Water samples for analysis of Ti from TiO2 NPs were digested with ammonium persulfate and dissolved in 2% HNO3 to yield Ti4+ ion using the method of Khosravi et al. (2011). Water samples McGeer containing TiO2 NPs were diluted and transferred to porcelain annealing cups. Samples were evaporated at 80°C for 1 hour until completely dry. Ammonium persulfate (1 gram) was placed in each dry annealing cup and spread to cover the bottom of the cup completely. Annealing cups were then suspended over a Bunsen burner (using a wire mesh) until fuming ceased (approx. 15 min), at which point [TiO(SO4)2] has formed. Cups were cooled at room temperature, then 5 mL of 2% nitric acid (trace metals grade, Fisher Scientific, Mississauga ON) was added along with a micro stir bar and then they were placed on a hot plate and the mixture gently boiled for approximately 10 minutes. The resulting solution with TiO2 NP converted to Ti4+ was then saved and subsequently analyzed by graphite furnace atomic absorption spectroscopy (GF-AAS).

Details on test solutions
Stock TiO2 NP solutions were made by adding 1g of TiO2 NP powder to 1L of test medium to yield final concentrations of 1g TiO2 /L. In order to achieve a monodispersed solution, NPs were placed in test media and were dispersed in a two step method. The first step involved mixing of stock solutions using a stir bar for 24h (Wiench et al., 2009). Secondly a sonication step was performed. 186 mL of stock solution were sonicated using a probe sonicator (QSonica, Sonicator 4000, Newton, CT) for 5 minutes at 20 kHz, 20mm, 0.5 inch Ti horn prior to addition into exposure system (Wiench et al., 2009, Termnak 2007).

Test organisms

Details on test organisms
H. azteca were removed from exposure system at the end of the exposure using a disposable pipette and placed in clean culture water. Organisms were given 6 hours for gut clearance, transferred and blotted dry before being placed (with a fine tip paint brush) in a 0.6 mL ultracentrifuge tube to be baked for 48 hours at 80°C. After drying was complete individual organisms were weighed using a Sartorious SE2 Ultra Micro Balance (Sartorius Mechantronics Corp., Bohemia, NY, U.S.A).

Study design
Test type
static
**Total exposure duration**
28 d

**Remarks**

**Test conditions**

**Hardness**
40 mg CaCO3/L

**Test temperature**
22°C ± 1°C

**pH**
7.3 ± 0.1

**Salinity**
0.31mM CaCl2-2H2O, 0.31mM NaHCO3, 0.003mM NaBr, 0.02mM KCl, and 0.08mM of MgSO4-7H2O (Sigma-Aldrich Inc. St. Louis, MO)

**Nominal and measured concentrations**
dissolved Ti: nominal concentrations of 0, 0.1, 0.3, 0.75, 1.5 and 3 mg/L TiO2-NP: nominal concentrations of 0, 1, 5, 10, 20, 50 and 100 mg/L

**Details on test conditions**

An initial H. azteca culture was obtained from Aquatic Research Organisms (ARO; Hampton, NH, U.S.A.) and cultured following protocols from Borgmann (2002). An artificial culture medium was used and made with deionized water to obtain a hardness of 130 mg CaCO3/L (1mM CaCl2-2H2O, 1mM NaHCO3, 0.01mM NaBr, 0.05mM KCl, and 0.25mM of MgSO4-7H2O. A 24h presoaked sterile piece of cotton gauze (5 cm X 5 cm) was placed in each beaker as a substrate for the H. azteca. Temperature was held at 22°C ± 1 °C with 16h light and 8h dark photoperiod. Tetramin™ flakes (Tetra Werke, Blacksburg, VA, U.S.A.) were ground up and passed through 500μm sieve, organisms received 5mg of dry Tetramin™ flakes 3 times per week. Water renewals were done weekly. H. azteca chronic toxicity tests (28d) were carried out according to EPS/11RM/33. Exposure conditions were maintained at 22°C ± 1oC with 16h light and 8h dark photoperiod. A 5 cm X 5 cm piece of cotton gauze was used as substrate and each beaker received 5mg of dry Tetramin™ flakes 3 times per week. Organisms were 2-9 d of age when tests began. Test media was made by dissolving 0.31mM CaCl2-2H2O, 0.31mM NaHCO3, 0.003mM NaBr, 0.02mM KCl, and 0.08mM of MgSO4-7H2O (Sigma-Aldrich Inc. St. Louis, MO) with a pH of 7.3 ± 0.1 and a final hardness of 40 mg CaCO3/L. Exposures were done in duplicate and were static renewal tests with 100% of water volume being replaced weekly. Polypropylene beakers were used for exposures and held 400mL of spiked medium. Twenty H. azteca of 2 – 9d of age were exposed to Ti from AAS standards (Sigma-Aldrich Inc. St. Louis, MO), which is called ‘dissolved Ti’ at nominal concentrations of 0, 0.1, 0.3, 0.75, 1.5 and 3 mg/L in duplicate. Water was spiked with dissolved Ti and pH was adjusted as needed with 1M KOH solution made by dissolving KOH pellets (Sigma-Aldrich Inc. St. Louis, MO) in MilliQ ultrapure water. Twenty H. azteca 2 – 9d of age were exposed to TiO2 NPs at nominal concentrations of 0, 1, 5, 10, 20, 50 and 100 mg/L in duplicate, with NP details on Table 2.1. Stock TiO2 NP solutions were made by adding 1g of TiO2 NP powder to 1L of test medium to yield final concentrations of 1g TiO2 /L. Exposures were all in static renewal with 100% water changes performed weekly.

**Reference substance (positive control)**
No
## Results and discussions

### Effect concentrations

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<thead>
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</tr>
</thead>
<tbody>
<tr>
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<td>Basis for effect</td>
<td>growth dry weight</td>
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<td>Remarks (e.g. 95% CL)</td>
<td>NM 101</td>
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<td>Remarks (e.g. 95% CL)</td>
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### Details on results

During 28d chronic exposures to dissolved Ti of nominal concentration of 300, 750, 1500 and 3000 µg Ti/L correspond to measured concentration of 278 ± 27.5, 501 ± 77.6, 595 ± 109, and 2349 ± 527 µg Ti/L respectively (n = 8). Survival decreased with increasing dissolved Ti exposure concentrations and an LC50 value of 1404 ± 347 µg Ti/L was calculated for dissolved Ti. There was significant impaired
growth based on dry weight per organism at exposure concentrations above 501 μg Ti /L of dissolved Ti. An IC50 of 914 ± 369 μg Ti /L was calculated. Hyalella azteca chronically (28d) exposed to sonicated solutions of NM101 TiO2 NPs of nominal concentration of 20, 50, and 100 mg TiO2 /L which correspond to measured concentration of 7.8 ± 1.6, 20.4 ± 1.6, and 48.6 ± 2.97 mg TiO2 /L respectively (n=8). An LC50 could not be calculated since exposure at tested concentration did not greatly impact survival. There was however significant reduction in dry weight with increasing TiO2 additions. IC50 value of 15.98 ± 1.4 mg TiO2 /L and an IC20 value of 8.8 ± 2.8 mg TiO2 /L were calculated.

Reported statistics and error estimates
Data are all expressed as mean ± 1 standard error of the mean (SEM) and statistical analysis was performed using SigmaPlot 11.0 computer software (Systat Software, Inc., San Jose, CA). Dry weight of organism during standard toxicity tests was subjected to a one-way analysis of variance (ANOVA) using Dunnet’s post hoc test to detect significant difference of dry weight relative to control (unexposed) groups. All effect concentration values were calculated using Spearman-Karber analysis using the Comprehensive Environmental Toxicity Information System software (CETIS V1.6.1 rev C) and statistical significance was taken as P<0.05. Note that not all TiO2 solutions were measured for Ti content. Only test solutions that bracketed the nominal effect concentration range were measured. There were no growth effects seen at nominal concentrations less than 20 mg/L. In each part of the results these nominal and measured concentrations are given.

6.1.5 Toxicity to aquatic algae and cyanobacteria

Endpoint study record: Pseudokirchneriella subcapitata (publication)

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result

Data source

Reference

Reference type publication
Author Hartmann,N.B., Von der Kammer F., Hofmann,T., Baalousha,M., Ottofuelling,S., and Baun,A.
Year 2010
Title Algal testing of titanium dioxide nanoparticles-Testing considerations, inhibitory effects and modification of cadmium bioavailability.
Bibliographic source Toxicology. 269, 190-197

Applicant's summary and conclusion

Executive summary
Test material: Hombikat UV100 Source type: publication (peer-review) Guideline: ISO 8692 (set-up in 4 mL) Organism: Pseudokirchneriella subcapitata Test media + conditions: ISO algal medium Study type: laboratory test Test duration: growth test Application method: stock suspension: 250 mg/L in test medium, 10 min sonication in water bath; stored at 5 °C in the dark and sonicated again (10 min) prior to test Endpoint: growth rate Chemical analysis, Material characterization: particles surface are (BET), TEM, SEM, DLS after 0, 2, 6, 48, 72 h; particle sizer to measure particles sizes above the size which can
be measured by DLS GLP: not specified Validity criteria according to the guideline fulfilled: no information Medium: ISO medium according to guideline Test concentrations: 16 test concentrations (0.6 – 250 mg/L) Suitability of applied methods: no obvious limitations Deviations from standard procedure: miniaturized set up (4 mL) Results: Hombikat UV100: EC10=3.3 mg/L (0.5-21) EC20= 14.5 mg/L (4.4-48) EC50=241 mg/L (96-609) (The EC50 is not reliable because of the large confidence interval.) Information concerning test and procedure: no information on CVs of control Remark: --- Conclusion - suitability of the study as input for DDP (adapted from Klimisch Code - report of ECETOC): 2c

6.1.6 Toxicity to aquatic plants other than algae

6.1.7 Toxicity to microorganisms

6.1.8 Toxicity to other aquatic organisms

*Endpoint study record: biofilm communities (publication)*

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Data source**

**Reference**

**Reference type** publication

**Author** Battin,T.J., Kammer,F., Weilhartner,A., Ottofuelling,S., and Hofmann,T. **Year** 2009

**Title** Nanostructured TiO2: Transport Behavior and Effects on Aquatic Microbial Communities under Environmental Conditions.

**Bibliographic source** Environmental Science & Technology 43, 8098-8104.

**Applicant's summary and conclusion**

**Executive summary**

Test material: Hombikat UV-100 Source type: publication (peer-review) Guideline: --- Organism: planktonic and biofilm communities Test media + conditions: General setup: natural surface water (chemically characterised) amended with nutrients and glucose was used Batch experiments: initial cell abundance after dilution of 9.6 x 10^7 cells/mL-1, three batches with 5.3 mg/L TiO2 and control batch were used; batches were operated under simulated light and in parallel under dark conditions, triplicate sample collection after injection and after 24 h Microcosm experiments: Microcosms set up according to literature and adapted, triplicate microcosms for each of the test materials, duplicate microcosm as control (without TiO2), 24 d biofilm growth on glass slides under simulated light, afterwards recirculation (water depth 15 mm, volume 1400 ml, concentration of TiO2 5.3 mg/L), recirculation of TiO2 for 48 h under simulated light, triplicate slides collected after 4, 24, 48 h Study type: laboratory test Test duration: short term and long term Application method: as suspension in filtered (0.45 μm; cellulose acetate membrane) surface water (Lunzer Untersee, Austria) Treatment: Ultrasonication (probe sonication) of suspension of TiO2-samples in water (30s, 25W at 10% energy input) Application: injection of probes into microcosms (biofilm) and batches (planktonic cells) Endpoint: cell membrane integrity, ROS detection Chemical analysis, Material characterization: yes: particle size distribution by DLS; TEM GLP: not specified
Validity criteria according to the guideline fulfilled: ---- (no guideline study) Medium: natural surface water Test concentrations: 5.3 mg/L Suitability of applied methods: no obvious limitations Deviations from standard procedure: ---- (no guideline study) Results: The results suggest adverse effects are not necessarily only attributable to individual particles smaller than 100 nm but also to low concentrations of larger, naturally agglomerating TiO2 nanoparticles. Cell membrane damage was more pronounced in free-living cells than in biofilm cells, indicating the protective role of cell encapsulation against TiO2 nanoparticles. The generation of intracellular reactive oxygen species (ROS) further suggests nano-TiO2-induced effects inside the microbial cells. Information concerning test and procedure: Is the information comprehensively and sufficiently: yes Remark: Homogenisation of biofilms when measuring membrane damage destroys vertical distribution; probably only the upper membranes were damaged. Conclusion that nano-TiO2 has significant impact on microbial communities when based only on these findings seems premature, esp. since the action mechanism of ROS-generation was not determined reliably – adapted from Klimisch code (ECETOC report): 2e

**Endpoint study record: Activated sewage sludge by RWTH-Aachen**

**Administrative Data**

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<td>Rationale for reliability</td>
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**Data source**

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<td>Author</td>
<td>Anne Wyrwoll</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Testing laboratory</td>
<td>Institute for Environmental Research, RWTH-Aachen University, Germany</td>
</tr>
<tr>
<td>Owner company</td>
<td>Federal Environment Agency Germany ; Institute of Environmental Research, RWTH-Aachen University, Germany</td>
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**Data access**

data submitter is data owner

**Cross-reference to same study**
The same study was performed with the nanomaterial NM 102 and with the non nano scale titanium dioxide material NM 100.

**Materials and methods**

**Test guideline**

<table>
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<th>Qualifier</th>
<th>according to</th>
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<tbody>
<tr>
<td>Guideline</td>
<td>other guideline: OEDD 209 Activated sludge respiration inhibition test</td>
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</table>

**GLP compliance**

no in the style of GLP
Test materials
Analytical monitoring
no

Test organisms
Test organisms (species)
other: Activated sludge

Details on test organisms
Activated sludge, microorganisms from a domestic waste water treatment plant were supplied by a municipal sewage treatment plant (Bensheim, Germany). The activated sludge used for this study was used as collected, but coarse particles were removed by settling for a short period (e.g. 5 – 15 minutes). Thereafter, the upper layer was decanted.

Study design
Test type
Static

Water media type
freshwater

Total exposure duration
3 h Remarks

Test conditions
Reference substance (positive control)
yes 3,5-dichlorophenol

Any other information on materials and methods incl. tables
The test was performed according to the OECD guideline 209. More precisely a preliminary test was conducted in which three different concentrations of the nanomaterial (10, 100, 1000 mg/L) and a blank control were tested. By addition of N-allylthiourea to additional controls and the highest concentration of the nanomaterial it was tested whether the activated sludge nitrifies and if so whether the nitrification was affected by the nanomaterial. Each treatment group consisted of three replicates. Every test stock suspension (1 g/L in deionized water) was prepared as explained in the standard operating procedure (SOP) "Preparation of a NM 101-suspension". Thereafter, different volumes of the stock suspension were added to the specific test medium containing tap water and activated sludge. Care was taken that the volume of deionized water was the same in every treatment group and in the control group.

Results and discussions
Effect concentrations

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<tr>
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Nominal/Measured
Conc. based on
Basis for effect
Remarks (e.g. 95% CL)
Duration
Endpoint
Effect conc.
Nominal/Measured
Conc. based on
Basis for effect
Remarks (e.g. 95% CL)

**Details on results**
The controls fulfilled the validity criteria of the guideline. NM 101 did not affect the heterotrophic activated sludge respiration nor the nitrification, therefore no effective concentrations (ECx) were calculated. The lowest observed effect concentration is higher and the no observed effect concentration is equal to or higher than 1000 mg/L.

**Results with reference substance (positive control)**
3,5-Dichlorophenol was used as reference substance (3.2, 10 and 32 mg/L). The EC50 accounted to 3.5 mg/L and was within the range stated in the OECD guideline (2-25 mg/L).

**Overall remarks, attachments**
Attached background material
Attached document 2: SOP Preparation of a NM 101 suspension.pdf:
ENV/JM/MONO(2015)17/PART3/ANN1

**Applicant’s summary and conclusion**
Validity criteria fulfilled
Yes

**Conclusions**
NM 101 did not affect the respiration of the activated sludge under the test conditions used in this study.

**6.2 Sediment toxicity**

*Endpoint study record: Chironomids.by Fraunhofer Institute for Molecular Biology and Applied Ecology (IME)*

**Administrative Data**
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Study period May 27 - June 24, 2010
Reliability 1 (reliable without restriction)
Rationale for reliability  Guideline study; following GLP; validity criteria fulfilled

Data source

Reference

Reference type  study report
Author  Hund-Rinke Kerstin; Klawonn Thorsten
Year  2012
Title  Emergence test with chironomids In: Investigation of two widely used nanomaterials (TiO2, Ag) in standardized ecotoxicological tests
Bibliographic source  Report for German Environment Agency
Testing laboratory  Fraunhofer Institute for Molecular Biology and Applied Ecology
Report no.  3709 65 416

Materials and methods

Test guideline
Qualifier  according to
Guideline  OECD Guideline 219 (Sediment-Water Chironomid Toxicity Test Using Spiked Water)
Deviations  no

GLP compliance
yes (incl. certificate) Certificate attached; concerning GLP see explanation in "Overall remarks"

Test materials
Test material equivalent to submission substance identity
Yes

Analytical monitoring
yes

Details on sampling
For the control and for each concentration one additional vessel was used especially for analytical measurements. The additional vessels were treated as the control vessels and the test vessels used for the assessment of the nanomaterials. At several points of time aqueous samples (50 mL) were taken at four depths (about 2.0 cm; 4.0 cm; 5.5 cm; 6.5 cm). The samples were combined. About 20 mL were used for analysis and the remaining amount was carefully returned into the test vessels without disturbing the sediment.

Details on analytical methods
Characterization of application dispersion and test dispersion Chemical analysis was performed in the samples collected from the additional vessels. Furthermore, using a Malvern Zetasizer the Zeta potential was measured in one vessel of each concentration and of the control three hours after application of the nanoparticles. Particle size distribution was determined in the control and the test vessels with P25 and NM-300K at selected time points during the incubation period. No measurements were performed in the highly concentrated application dispersions as the particle size distributions were not representative for the particle size distribution in the test vessels Physical-chemical parameters (overlaying water) In all vessels temperature and pH were measured at test start and test end as well as once a week during the study. Dissolved oxygen was measured in one representative vessel per treatment at test start and twice a
week during the course of the study, and in all test vessels at the end of the test. Hardness and ammonia were measured in the controls and at the highest concentration in one test vessel at the start and the end of the study. Further details are reported in the attached file.

**Vehicle**

no

**Details on sediment and application**

The nominal concentrations in the test containers with the test item were 15, 23, 39, 63, and 100 mg test item/L. Four replicates per concentration were conducted. For each vessel a 500 mL stock dispersion of the nanomaterial was prepared in tap water. For the double concentrated dispersion of the final test concentration the respective amount of nanomaterial was weighted in brown glass vessels using a suitable balance. 500 mL of tap water was added, the mixture was stirred (magnetic stirrer, 900 rpm), followed by ultrasonic treatment in a water bath (3 min, 900 W). The stock dispersion was added thoroughly to the water column in the test vessels 24 h after adding the test specimens. Due to the large amount of stock dispersion the dispersion immixed while being added to the water column. There was no further mixing to avoid a disturbance of the sediment.

**Test organisms**

**Test organisms (species)**

Chironomus riparius

**Details on test organisms**

Test organisms were the first instar larvae from the dipteran Chironomus riparius. Origin of the midges: Bayer Crop Science AG, 40789 Monheim, Germany. Specimens used in the test were bred in the laboratory of the Fraunhofer IME. Breeding conditions: Purified tap water was added to a layer of diatomaceous earth. The dipterans were fed daily with powder of TetraMin® Hauptfutter (Tetra Werke, Melle, Germany). Pre-treatment: Four to five days before adding the test organisms to the test vessels egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20°C. First instar larvae (one day post hatching) were used in the test. As the larvae were added one day before spiking, the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase).

**Study design**

**Study type**

laboratory study

**Test duration type**

long-term toxicity

**Test type**

static

**Water media type**

freshwater

**Type of sediment**

artificial sediment

**Limit test**

no
Total exposure duration
28 d

Remarks
Post exposure observation period
no

Test conditions

Hardness
Test start 130 – 150 mg/L as CaCO₃ equivalents and 140 mg/L as CaCO₃ equivalents in one representative replicate of the highest test concentration (demanded threshold value of 400 mg/L as CaCO₃ equivalents) Test end: 150 – 170 mg/L as CaCO₃ equivalents in the controls and 170 mg/L as CaCO₃ equivalents in one representative replicate of the highest test concentration.

Test temperature
20.3 °C - 20.5 °C (permitted range: 20 ± 2 °C)

pH
7.8 – 8.7 (permitted range: pH 6 – 9)

Dissolved oxygen
About 100% at test start and test end (demanded threshold value: 60%)

Ammonia
Test start: 0.5 - 0.9 (control); 0.7 (highest test concent-ration) Test end: 0.1 - 7.5 (control); 0.6 (highest test concent-ration)

Nominal and measured concentrations
The nominal concentrations in the test containers with TiO₂ were 15, 23, 39, 63, and 100 mg test item/L.

Details on test conditions
The light intensity was measured using an illuminance meter (MINOLTA) with photo-metric sensor in Lux. With 771 – 826 lx the permitted range of about 500 – 1000 lx was kept.

Reference substance (positive control)
no

Any other information on materials and methods incl. tables
Control treatment The control consists of sediment, tap water and chironomids. Four replicates per control were conducted. Statistical method Data evaluation: Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with the rounded values compared to the values obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern. Statistical calculations: Calculations were performed with the computer software ToxRat Professional version 2.10.4.1 by ToxRat® Solutions GmbH. Food Powder of TetraMin® Hauptfutter was used for feeding the larvae. According to the guideline the food ration for the first 10 days was 0.25 – 0.5 mg TetraMin® /larvae/day, from day 10 on the food ration was increased to 0.5 – 1.0 mg TetraMin® /larvae/day. Test container Round glass beakers (3L) were used as test vessels. The vessels were filled up with wet artificial sediment (corresponding to 370 g dry mass). The height was 2 cm. The overlaying water was 8 cm high (ratio sediment : water about 1:4). The con-tainers were covered with glass plates. After 10 days, emergence traps were placed on the test vessels, the glass plates remained on the emergence traps to avoid evaporation. Aeration of overlaying water was provided through a glass pipette fixed 2-3 cm above the sediment layer (at least 1
bubble /second). Test procedure Sediment was filled in the test vessels. 400 mL of tap water was added and the sediment-water system was left under gentle aeration for several days prior to adding the test organisms. Batches of twenty larvae were placed into each vessel. After incubation for 24 h, 500 mL of the freshly prepared stock dispersion of the nanomaterials was added. Further 100 mL of tap water were used to rinse the vessels containing the stock dispersions. To avoid separation of sediment ingredients during addition of test water and stock dispersion, the surface of the water column was covered with a stainless steel disc while water was poured onto it. The disc was removed immediately afterwards. Due to the large amount of stock dispersion the dispersion immixed while being added to the water column. There was no further mixing to avoid disturbance of the sediment. The test was carried out at 20 °C ± 2 °C and at 16 hours photoperiod (500 –1000 lux). The exposure duration was 28 days. Development time and total number of fully emerged male and female midges were determined. Test vessels were observed daily for visual assessment of abnormal behaviour. Emergence was counted daily. After identification the midges were removed from the test vessel. At test end, the test vessels were observed for visible pupae that had failed to emerge.

Results and discussions

Effect concentrations

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<td>Basis for effect</td>
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Remarks (e.g. 95% CL)

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</tr>
<tr>
<td>Basis for effect</td>
<td>development rate</td>
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</table>

Details on results

for details and raw data see attached document

Reported statistics and error estimates

Data evaluation: Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with the rounded values compared to the values obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern. Statistical calculations: Calculations were performed with the computer software ToxRat Professional version 2.10.4.1 by ToxRat® Solutions GmbH.
Overall remarks, attachments

Overall remarks
The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data is performed and the quality assurance unit was not involved with respect to the inspection of the test, of the raw data and the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

Attached background material

Attached full study report

Applicant's summary and conclusion

Validity criteria fulfilled
yes The test is considered valid since: • The mean emergence in the controls was 92.5% (corresponding to 70% mentioned in the guideline) at test end. Furthermore: • The development time of the adults of C. riparius in the controls was between 16 and 21 days

Conclusions
NM-101: Up to a concentration of 100 mg/L NM-101 resulted in no negative impact on the emergence of larvae in a spiked water-sediment test with chironomids. The NOEC is ≥ 100 mg/L.

Executive summary
TiO2 was tested in the test with chironomids (Chironomus riparius) with spiked water (OECD 219). The nominal concentrations in the test containers with TiO2 were 15, 23, 39, 63, and 100 mg test item/L. There was strong sedimentation of TiO2 resulting in Ti concentrations in the overlaying water below the detection limit. At test end nearly all of the TiO2 was determined in the sediment. NM-101: Up to a concentration of 100 mg/L NM-101 resulted in no negative impact on the emergence of larvae in a spiked water-sediment test with chironomids. The NOEC is ≥ 100 mg/L.

6.3 Terrestrial toxicity

6.3.1 Toxicity to soil macroorganisms except arthropods

Endpoint study record: Toxicity to soil macroorganisms except arthropods.001 by RWTH-Aachen

Administrative Data
Purpose flag key study ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Study period November 2010 - June 2013
Data source

Reference
Reference type study report
Author Anne Wyrwoll
Testing laboratory IBACON GmbH
Owner company UBA, IBACON GmbH, RWTH Aachen

Data access
data submitter is data owner

Data protection claimed
yes

Materials and methods

Test guideline
Qualifier according to
Guideline OECD Guideline 207 (Earthworm, Acute Toxicity Tests)
Deviations

Test materials

Analytical monitoring
yes

Details on sampling
Soil samples were collected at the end of the experiment. Subsequently they were frozen at -20°C until analysis. Prior to analysis samples were dried in an oven for 15 h at 105°C. Dried samples were than ground finely with a ball mill.
Details on analytical methods
Ground soil samples were digested with a mixture of hydrofluoric acid, nitric acid and perchloric acid. Thereafter they were analyzed with inductive coupled plasma optical emission spectroscopy (ICP-OES, LOQ 5 ppb). Access to titanium analysis provided at the Natural History Museum London within the QualityNano scheme funded by the European Commission under FP7 Capacities Programme Grant Agreement No: 262163

Details on preparation and application of test substrate
See attached standard operating procedure 'Application of a nanomaterial-suspension to soil'

Test organisms

Test organisms (species)
Eisenia fetida

Animal group
annelids
Study design

Study type
laboratory study

Test duration type
short-term toxicity

Substrate type
natural soil

Limit test
yes

Total exposure duration
14

Remarks
Post exposure observation period
1 day post exposure on wet paper to defecate

Test conditions
Test temperature
20°C

pH
5

Moisture
55% of maximum water holding capacity of the test soil

Nominal and measured concentrations
Nominal: 1000 mg/kg Measured: 966 mg/kg. This is 97% of the nominal value.

Details on test conditions
Test soil: Natural reference soil RefeSol 01-A a natural, slightly loamy, middle acidic, very slightly humic soil was used as test soil. The soil was air dried, sieved through a 2 mm sieve and stored at room temperature until use.

Results and discussions

Effect concentrations

<table>
<thead>
<tr>
<th>Duration</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoint</td>
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</tr>
<tr>
<td>Effect conc.</td>
<td>&gt;= 1000</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
<tr>
<td>Conc. based on</td>
<td>test mat.</td>
</tr>
<tr>
<td>Basis for effect</td>
<td>mortality</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td></td>
</tr>
</tbody>
</table>
Overall remarks, attachments

Attached background material


Applicant's summary and conclusion

Validity criteria fulfilled
yes

Conclusions
At a level of 1000 mg/kg NM 101 had no effect on the mortality of Eisenia fetida. The results of the titanium analysis of the test soils show that the application method described in the attached standard operating procedure resulted in a homogeneous and reproducible application of the TiO2 nanomaterial to the test soil.

Endpoint study record: earthworm reproduction. Fraunhofer Institute for Molecular Biology and Applied Ecology(IME)

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Study period 1st test: February 18 - April 14, 2010; 2nd test: January 21 - March 18, 2011
Reliability 1 (reliable without restriction)
Rationale for reliability Guideline study; following GLP; validity criteria fulfilled

Data source

Reference
Reference type study report
Author Hund-Rinke Kerstin; Klawonn Thorsten
Year 2012
Title Earthworm reproduction In: Investigation of two widely used nanomaterials (TiO2, Ag) in standardized ecotoxicological tests
Bibliographic source Report to German Environment Agency
Testing laboratory Fraunhofer Institute for Molecular Biology and Applied Ecology
Report no. 3709 65 416

Materials and methods

Test guideline
Qualifier according to
Guideline OECD Guideline 222 (Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei))
Deviations no
GLP compliance
yes (incl. certificate) Certificate attached; concerning GLP see explanation in "Overall remarks"

Test materials
Test material equivalent to submission substance identity
yes
Test material identity
Identifier
Identity NM-101

Analytical monitoring
yes

Details on analytical methods
Due to the high natural concentration of TiO2 in the test soil no specific chemical analyses were performed in this medium. Zeta potential was measured in the test dispersions using a Zetasizer Nano ZS. Following instrument settings were applied: (I) refractive index: 2.55; (II) adsorption: 0.073. The particle size distribution was not determined: (i) the high concentrations of TiO2 precluded a determination of the particle size distribution, (ii) size distribution in the dispersion would give no information on the size distribution in soil or feed. At present, measuring the Zeta potential or particle size distribution in soil is not possible. TiO2 was determined in the earthworms. Earthworms were incubated for 24 h on wet filter paper to purge their gut. Afterwards they were frozen (-20 °C) until analysis.

Details on preparation and application of test substrate
We tested four different modes of application. Spiking of soil with TiO2 powder For the first application the TiO2 powder was mixed directly into the soil, whereby air-dried test soil (1% of the total amount) was used as a carrier. Suitable amounts of TiO2 powder to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO2 crystalline structure. Uncontaminated test soil (between 20 and 30% of WHCmax) was spread on a plate, the carrier material with the TiO2 powder was distributed on the test soil, and all was mixed carefully. For the test with contaminated soil, the soil was adjusted to a water-holding capacity of 55% of the maximum water-holding capacity (WHCmax). Test concentrations were: 50, 100 and 200 mg/kg soil dry matter (d.m.). Spiking of feed with TiO2 powder The second application trial was the direct introduction of TiO2 into the earthworm feed, which consisted of antibiotic-free cow manure. In all four replicates, 40 g of air-dried ground cow manure were homogenously mixed with TiO2 powder. The mixture was moistened with 120 ml deionized water. Test concentrations were: 3.19, 6.38 and 12.76 mg/g feed (d.m.) corresponding to 50, 100 and 200 mg/kg soil (d.m.); 40 g moist feed (10 g dry feed and 30 ml deionized H2O) were applied on the surface of the 1-L test containers, each of which was filled with 640 g soil (d.m.). Spiking of soil with aqueous TiO2 dispersion The third application trial was to spray a TiO2 dispersion that had been prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. Test soil was dried to about 10% of WHCmax and spread on a plate. Immediately after preparation a predetermined amount of the highly concentrated TiO2 dispersion was sprayed onto the soil by means of a syringe coupled with a cannula, and then thoroughly mixed. Finally, the test soil was adjusted to a water-holding capacity of 55% of WHCmax. A maximum concentration of 200 mg/L application dispersion of TiO2 nanoparticles was considered adequate for the tests. Higher concentrations would have sedimented rapidly preventing a homogenous distribution of the nanomaterial in the soil. Maximum water content in the test soil should be about 55% of the maximum water-holding capacity. Due to these limitations, only soil contents of 10 and 20 mg/kg were tested. Test concentrations were: dispersion with 100 and 200 mg/L deionized water; application of 250 ml test dispersion to 2.5 kg test soil (d.m.) corresponding to 10 and 20 mg/kg soil (d.m.). Spiking of feed with aqueous TiO2 dispersion The fourth
and final type of application was a mixture of TiO2 dispersion and earthworm feed, whereby 40 g of cow manure was mixed with 120 ml concentrated TiO2 dispersion. Test concentrations were: dispersion with 212 and 424 mg/L deionized water; application of 120 ml test dispersion corresponding to 10 and 20 mg/kg soil (d.m.)

Test organisms
Test organisms (species)
other: Eisenia andrei

Animal group
annelids

Details on test organisms
The test organisms were synchronized adult earthworms of the species Eisenia fetida andrei (Annelida, Oligochaeta), which were 2 - 12 months old, with a clitellum, and a wet mass between 250 mg and 600 mg. Origin of the worms: Regenwurmfarm Tacke, Klosterdiek 61, 46325 Borken. Specimens used in the test were bred in the laboratory of the Fraunhofer IME. Breeding conditions: Worms were bred in 1:1 mixtures of cow manure and Sphagnum peat (dry mass basis) at 20 °C ± 2 °C. Pre-treatment: The worms were conditioned in the artificial soil for 7 days before use. The same feed as used in the test (see 9.3) was given in a sufficient amount.

Study design
Study type
laboratory study

Test duration type
long-term toxicity

Substrate type
natural soil

Limit test
no

Total exposure duration
56 d

Remarks
Post exposure observation period
no

Test conditions
Test temperature
The incubation temperature was measured continuously with a thermograph. According to the guideline the permitted range is 20 ± 2 °C.

pH
5.6
Moisture
55% of the maximum water holding capacity

Nominal and measured concentrations
TiO2 The following nominal contents were applied in the test containers with TiO2: 50, 100, 200 mg/kg soil, dry mass (application via powder on soil) 50, 100, 200 mg/kg soil, dry mass (application via powder on feed) 10, 20 mg/kg soil, dry mass (application via dispersion on soil) 10, 20 mg/kg soil, dry mass (application via dispersion on feed) The following concentrations were investigated in the second test with: 50, 100, 200, 400 mg/kg soil, dry mass (application via powder on soil).

Details on test conditions
The incubation temperature was measured continuously with a thermograph. According to the guideline the permitted range is 20 ± 2 °C. A controlled light/dark cycle of 16 h : 8 h was applied. The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. According to the guideline the permitted value is about 600 lx. The test conditions are presented in Table 1.

<table>
<thead>
<tr>
<th>Incubation temperature [°C]</th>
<th>19 – 21</th>
<th>19 – 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity [lx]</td>
<td>600 – 750</td>
<td>600 – 800</td>
</tr>
<tr>
<td>Soil dry mass [%]</td>
<td>79 – 90</td>
<td>81 – 89</td>
</tr>
<tr>
<td>pH (1 mol/L KCl) – test start</td>
<td>4.8 – 4.9</td>
<td>5.0</td>
</tr>
<tr>
<td>pH (1 mol/L KCl) – test end</td>
<td>6.2 – 6.4</td>
<td>6.7 – 6.9</td>
</tr>
</tbody>
</table>

Reference substance (positive control)
yes Carbendazim

Any other information on materials and methods incl. tables
Frequency of treatment Treatment was performed once at test start. Control group and treatment For TiO2 the control consists of soil. Eight replicates per control were conducted. Statistical method Data evaluation In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with rounded values compared to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern. Statistical calculations For each concentration the percent mortality, the percent loss/increase in biomass of the adults, and the number of offspring produced in the test was determined. Means were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. Statistical calculations were done with ToxRat® Pro 2.10, statistics software for ecotoxicity response analysis by ToxRat® Solutions. Feed Air-dried, finely ground cow manure was used as feed. Test container II tests were performed in polypropylene containers (Bellaplast GmbH, Alf). Adjusted to 55% of the maximum water-holding capacity, 640 g soil (d.m.) was filled in containers to a depth of about 5 cm. The containers were covered with transparent plastic lids to prevent worms from escaping and to guarantee access of light. The lids had several small holes to permit gaseous exchange between the medium and the atmosphere. Test procedure Soil and food were spiked. Test soil was filled in the test containers and an amount of 10 g air dried, finely ground cow manure per test container was spread on the soil surface and moistened with water. The next day (start of the test) batches of ten conditioned worms were weighed and placed into each container. Spiking of soil and food, respectively, filling of the test vessels and addition of the earthworms could not be performed at the same day due to high number of test variables and test concentrations. Once a week the worms were fed according to their feed consumption. Feeding behaviour and the quantity of feed applied over the test period was recorded for each test container. The water content of the soil substrate in the test containers was maintained during the test period by weighing the test containers periodically and replenishing loss of water, if necessary. The adult worms were kept in the substrate over a period of 4 weeks. At the end of this period, the adults were removed. For each container the total number and mass of living adult worms was recorded. To allow the offspring to develop, the test containers were kept in the test environment for another period of 4 weeks. After this...
period the number of offspring per test container hatched from the cocoons was counted by hand selection. The test was carried out at 20 °C ± 2 °C and a controlled light/dark cycle of 16 h : 8 h with a light intensity of 400 lx to 800 lx.

**Results and discussions**

**Effect concentrations**

<table>
<thead>
<tr>
<th>Duration</th>
<th>56 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>&gt;= 200 mg/kg soil dw</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
<tr>
<td>Conc. based on</td>
<td>test mat.</td>
</tr>
<tr>
<td>Basis for effect</td>
<td>reproduction</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td>1 st test; application via powder on feed</td>
</tr>
<tr>
<td>Duration</td>
<td>56 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>100 mg/kg soil dw</td>
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<tr>
<td>Nominal/Measured</td>
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<tr>
<td>Conc. based on</td>
<td>test mat.</td>
</tr>
<tr>
<td>Basis for effect</td>
<td>reproduction</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
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<td>Duration</td>
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<tr>
<td>Endpoint</td>
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<tr>
<td>Effect conc.</td>
<td>&gt;= 200 mg/kg soil dw</td>
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<tr>
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<tr>
<td>Basis for effect</td>
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<tr>
<td>Remarks (e.g. 95% CL)</td>
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<tr>
<td>Duration</td>
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<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>10 mg/kg soil dw</td>
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<td>Conc. based on</td>
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</tr>
<tr>
<td>Basis for effect</td>
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</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
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<tr>
<td>Duration</td>
<td>56 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
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<tr>
<td>Effect conc.</td>
<td>&lt; 10 mg/kg soil dw</td>
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<tr>
<td>-------------</td>
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<tr>
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</tr>
<tr>
<td>Conc. based on</td>
<td>test mat.</td>
</tr>
<tr>
<td>Basis for effect</td>
<td>growth</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td>1st test; application via dispersion on soil</td>
</tr>
<tr>
<td>Duration</td>
<td>56 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>&gt;= 400 mg/kg soil dw</td>
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<tr>
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<tr>
<td>Basis for effect</td>
<td>growth</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td>2nd test; application via powder on soil; measurement of biomass</td>
</tr>
</tbody>
</table>

**Details on results**

Raw data are included in the attached document. In some of the tests, the Ti concentration in earthworms was determined. There are strong indications that Ti concentrations in the earthworms increase with increasing test concentrations. However, there seems to be a difference depending on whether the contamination is highly concentrated in food or distributed in soil. Contaminated food seems to result in higher concentrations in the earthworms showing an increase already at concentrations of 100 or 200 mg/kg, whereas for contaminated soil an increase is obvious only for a concentration of 1000 mg/kg. Obvious differences between the three nanomaterials were not observed. As only two replicates were carried out, no calculation was performed concerning the statistical difference. In none of the test designs the concentration in the worms increases the soil/food concentration. Therefore, it is concluded that no accumulation in the worm tissue occurred and that the measured Ti was still in the gut, possibly attached to remaining soil/food particles.

**Results with reference substance (positive control)**

As reference substance Carbendazim was tested. Test period: February 11, 2010 - April 8, 2010 The following values were calculated for reproduction [mg/kg]: EC10: 1.147 (1.118 -1.172) EC20: 1.309 (1.289 -1.328) EC50: 1.688 (1.670 -1.709) LOEC: 1.500 NOEC: 0.750 All validity criteria were fulfilled. According to the guideline significant effects should be observed between 1 and 5 mg/kg. This criterion is fulfilled.
Reported statistics and error estimates
Data evaluation In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with rounded values compared to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern. Statistical calculations For each concentration the percent mortality, the percent loss/increase in biomass of the adults, and the number of offspring produced in the test was determined. Means were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. Statistical calculations were done with ToxRat® Pro 2.10, statistics software for ecotoxicity response analysis by ToxRat® Solutions.

Remarks on results including tables and figures
for raw data see attached document

Conclusions
The tested TiO2 nanoparticles did not cause a reduced number of offspring, but a stimulation was observed for P25 and NM-101 (uncoated nanomaterials) when the tests were performed in natural soil and in winter time. While the control showed a reduced number of offspring in winter compared to experiments carried out in summer, a reduced number of juveniles was not observed in the tests with P25 and in one test with NM-101. As the number of juveniles was not reduced in winter the number of juveniles was higher in the treated samples than in the control. On the basis of percent deviation of the treated samples compared to the control the higher number of juveniles results in stimulation. There are indications that the stimulation observed for P25 and NM-101 (uncoated nanomaterials) is due to the disturbance of the biological clock. For the coated TiO2 NM-103 no difference to the control was observed. Concentration measurements of TiO2 in the worm after purging of the gut were performed. It is concluded that no accumulation in the worm tissue occurred and that the measured Ti was still in the gut, possibly attached to remaining soil/food particles.

Executive summary
TiO2 nanoparticles (NM 101, NM 103, and P25) were tested in the earthworm reproduction test. The particles were applied as powder and as aqueous dispersion in soil and in feed. As test substrate a natural sandy soil was used. The experiments were performed several times. The following test concentrations were investigated: • Application via powder on feed: 50, 100, 200 mg/kg soil, dry matter • Application via powder on soil: 50, 100, 200 mg/kg soil, dry matter • Application via dispersion on feed: 10, 20 mg/kg soil, dry matter • Application via dispersion on soil: 10, 20 mg/kg soil, dry matter In the tests using a higher number of concentrations performed only with powder-spiked soil these concentrations were applied: • Application via powder on soil: 50, 100, 200, 400 mg/kg soil, dry matter (NM-101, NM-103) • Application via powder on soil: 50, 100, 200, 500, 750, 1000 mg/kg soil, dry matter (P25). The tested TiO2 nanoparticles did not cause a reduced number of offspring. A stimulation compared to the control can be observed at least for the uncoated material P25 when the test is performed in winter. For the coated material NM-103 a stimulatory effect cannot be observed. The stimulatory effect is less pronounced for the second uncoated material (NM-101). There are indications that the stimulation is due to the disturbance of the biological clock. Concentration measurements of TiO2 in the worm after purging of the gut were performed. It is concluded that no accumulation in the worm tissue occurred and that the measured Ti was still in the gut, possibly attached to remaining soil/food particles.

Overall remarks, attachments

Overall remarks
The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data is performed and the quality assurance unit was not involved with respect to the inspection of the test, of
the raw data and the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP. The test was repeated several times. To explain the observed stimulation the tests with further TiO2 nanomaterials (NM-101 and NM-103) are necessary. Therefore, in the attached report the tests with all TiO2 nanomaterials are included.

**Attached background material**


**Attached full study report**


**Validity criteria fulfilled**

yes As reference substance Carbendazim was tested. Test period: February 11, 2010 - April, 8, 2010 The following values were calculated for reproduction [mg/kg]: EC10: 1.147 (1.118 -1.172) EC20: 1.309 (1.289 -1.328) EC50: 1.688 (1.670 -1.709) LOEC: 1.500 N

**Endpoint study record: earthworm reproduction, mortality, avoidance (publication)**

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Data source**

**Reference type** publication

**Author** McShane J., Sarrazin M., Whalen J.K., Hendershop W.J., Sunahara G.I.

**Year** 2011

**Title** Reproductive and behavioral responses of earthworms exposed to nano-sized titanium dioxide in soil.

**Bibliographic source** Environ. Toxicol. Chem. Doi 10.10002/etc.714

**Applicant's summary and conclusion**

**Executive summary**

Test material: B: Hombikat UV100 (OECD batch assumed) Source type: publication (peer-review) Guideline: reproduction: OECD TG No. 222 (2004), mortality OECD TG No. 207 (1984) avoidance: ISO 17512-1; (2008) Organism: E. fetida, E. andrei; the species were not mixed in culture and in trials Test media + conditions: mortality, reproduction: natural soil (sandy loam; 5 % organic matter; adjustment of pH (H2O) to 6.5 – 6.7) avoidance: artificial soil Study type: laboratory test Test duration: short term (mortality; avoidance) and long term (reproduction) Application method: dispersion: stock dispersion (250 g/250 mL); raising pH to 10 using NaOH (< 5 mL added); vortexed for 3 min; left to stabilize at 20 °C for 4 h; revortexed and subsamples were pipetted into polypropylene containers of water to yield dispersions with nominal concentration of 60 and 600 mg/L. Addition to 500 g or 100 g of air-dry soil; final nominal concentrations: 20 or 200 mg/kg TiO2. Total Na concentration added to soil
was below the level shown to affect reproduction and survival solid: 0.1 – 10 g of TiO2 powder was added to 500 – 1000 g batches of air dry soil, mixed on a rotary shaper at 6 rpm for 20 – 24 h. for the avoidance test 2.0 and 4.0 kg of soil was hand-mixed Endpoint: avoidance, mortality, reproduction, juvenile growth test Chemical analysis, Material characterization: Particle size by TEM; particle crystallinity by X-ray diffraction; agglomerate hydrated diameter and the point of zero charge (40 g/L): DLS (Malvern Zeta Sizer); BET: metal concentration: ICP-MS. GLP: not specified Validity criteria according to the guideline fulfilled: yes Test concentrations: reproduction including mortality (OECD 222; mortality determined after 28 d)): 200, 10,000 mg/kg mortality (OECD 207; mortality determined after 14 d): 20, 200 mg/kg avoidance: 100, 1,000, 10,000 mg/kg Suitability of applied methods: no obvious limitations Deviations from standard procedure: no Results: reproduction, mortality: no effect up to 10,000 mg/kg avoidance: no dose response effects – Hombikat 40 % avoidance (5,000 mg/kg), 24 % avoidance (10,000 mg/kg) Information concerning test and procedure / Is the information comprehensively and sufficiently: yes Remark: --- Reliability – (adapted from Klimisch code; ECETOC report) : Mortality, reproduction1b Avoidance: 2e supporting information

**Endpoint study record: Toxicity to soil macroorganisms except arthropods by RWTH-Aachen**

**Administrative Data**

| Purpose flag | key study () robust study summary () used for classification () used for MSDS |
| Study result type | experimental result |
| Reliability | 2 (reliable with restrictions) |
| Rationale for reliability | in the style of GLP |

**Data source**

**Reference**

**Author** Anne Wyrwoll  **Year**

**Testing laboratory** IBACON GmBH  **Report no.**

**Owner company** UBA, RWTH Aachen, IBACON GmbH

**Data access**

data submitters is data owner

**Materials and methods**

**Test guideline**

**Qualifier** according to

**Guideline** OECD Guideline 222 (Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei))

**Deviations** no

**GLP compliance**

no in the style of GLP

**Test organisms**

**Test organisms (species)**
Eisenia fetida

**Animal group**
annelids

**Study design**

**Study type**
laboratory study

**Test duration type**
long-term toxicity

**Substrate type**
natural soil

**Total exposure duration**
56 d

**Remarks**

**Test conditions**

**Test temperature**
20°C

**pH**
Test initiation: 4.7-5.0 Test termination: 5.5-6.3

**Moisture**
55% of maximum water holding capacity of the test soil

**Nominal and measured concentrations**
Nominal: 1000 mg/kg

**Details on test conditions**
Test soil: Natural reference soil RefeSol 01-A a natural, slightly loamy, middle acidic, very slightly humic soil was used as test soil. The soil was air dried, sieved through a 2 mm sieve and stored at room temperature until use.

**Results and discussions**

**Effect concentrations**

<table>
<thead>
<tr>
<th>Duration</th>
<th>56 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>&gt; 1000 mg/kg</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
</tbody>
</table>
Conc. based on test mat.
Basis for effect reproduction
Remarks (e.g. 95% CL)

Overall remarks, attachments

Attached background material

Applicant's summary and conclusion

Conclusions
At a level of 1000 mg/kg NM 101 had no effect on the reproduction of Eisenia fetida.

6.3.2 Toxicity to terrestrial arthropods

6.3.3 Toxicity to terrestrial plants

6.3.4 Toxicity to soil microorganisms

6.3.5 Toxicity to birds

6.3.6 Toxicity to other above-ground organisms

6.4 Biological effects monitoring

6.5 Biotransformation and kinetics

6.6 Additional ecotoxicological information
7. TOXICOLOGICAL INFORMATION

7.1 Toxicokinetics, metabolism and distribution

7.1.1 Basic toxicokinetics

*Endpoint study record: Basic toxicokinetics_NM 101_Gavage by NANOGENOTOX*

**Administrative Data**

<table>
<thead>
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<th>Purpose flag</th>
<th>Study result type</th>
<th>Study period</th>
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<td>( ) robust study summary</td>
<td>experimental result</td>
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**Data source**

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<tr>
<td>study report</td>
<td>W De Jong</td>
<td>2012</td>
<td>Deliverable 7: Identification of target organs and biodistribution including ADME parameters</td>
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**Testing laboratory**

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**Company study no.**

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**Data access**

other: Owner: NANOGENOTOX

**Data protection claimed**

yes, but willing to share

**Materials and methods**

**Type of method**

in vivo

**Test material equivalent to submission substance identity**

yes

**Reference Material/Nanomaterial and Sample identification number**

<table>
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<th>Identifier</th>
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<td>Reference Material/Nanomaterial</td>
<td>NM 101</td>
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Test material identity

Identifier  CAS number
Identity     13463-67-7
Identifier  EC number
Identity     236-675-5

Test animals

Species  rat
Strain  Wistar
Sex  male/female

Administration / exposure

Route of administration
oral: gavage

Vehicle
other: Rat Serum Albumin (RSA) 0.05% diluted (9:1) v/v in 10 x phosphate buffer pH 7.4.

Duration and frequency of treatment / exposure
Administration: repeated (on 5 consecutive days, day 1-5) Sampling time: day 6

Doses / concentrations
2.3 mg of TiO2 resulting in a dose of 10.2-11.4 mg/kg bw/d (male) and 13.1-15.2 mg/kg bw/d (female). 5 day cumulative dose: 51-57 mg/kg bw (male) and 65.5-76 mg/kg bw (female)

No. of animals per sex per dose
Treated Groups: 3 M + 3 FControl: vehicle 2 M + 3 F

Control animals
yes, concurrent vehicle

Details on dosing and sampling
Tissues Sampled: Gastrointestinal tract, liver, spleen, , lungs, lymph nodes (mesenteric and popliteal),

Results
no bioaccumulation potential based on study results

Overall remarks, attachments

Attached full study report

Attached document  D2_WP4_SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks  Dispersion protocol
Remarks
Data in the report and details protocol in annex

Conclusions
No evidence for uptake of NM-101 following gavage.

Cross-reference to other study

7.2 Acute Toxicity

7.2.1 Acute toxicity: oral

7.2.2 Acute toxicity: inhalation

Summary of the HH literature data status 03rd April 2014: Summary of acute toxicity data (inhalation route)

<table>
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<tr>
<th>Reference</th>
<th>Material/ Size</th>
<th>Test Organism (Strain)/ Test System</th>
<th>Method</th>
<th>Exposure/ dose</th>
<th>Main findings</th>
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<td>Acute toxicity</td>
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<tr>
<td>Besov et al. 2010</td>
<td>8 nm anatase (Hombikat UV 100)</td>
<td>Mouse (Tomsk State University, outbred)</td>
<td>Inhalation</td>
<td>~ 4g/15 min.</td>
<td>Disturbed and aggressive behavior until 5 h after exposure.</td>
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Endpoint study record: Intratracheal instillation by Fraunhofer -ITEM

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Study period early 2013

Data source

Data access
other: performed and provided by Fh-ITEM

Cross-reference to same study
ITEM Study No. 02 N12 516
Materials and methods

Test type
other: Intratracheal instillation

Principles of method if other than guideline

Test animals
Species
rat

Strain
Wistar

Sex
male

Administration / exposure
Details on inhalation exposure
According to D. Schaudien, J. W. Knebel, I. Mangelsdorf, J.-U. Voss, W. Koch, O. Creutzenberg "Dispersion and Retention of Dusts Consisting of Ultrafine Primary Particles in Lungs" but using ultrasound with higher dose instead of UltraTurrax. total dose: 1.5mg/lung

Concentrations
4-wk Intratracheal Instillation Study with subsequent bronchoalveolar lavage (BAL) on days 3 and 27 1.5mg/rat Administration of total dose in two aliquots on consecutive days (day -2, day -1). Total dose given in two aliquots on two consecutive days, each suspended in 0.3 ml saline as mention into the referent publication for the method used.

No. of animals per sex per dose
5 ->day 3 5 ->day 27

Control animals
yes

Results and discussions
Preliminary study (if fixed dose study)
see attached document

Overall remarks, attachments
Attached background material
Applicant's summary and conclusion

Conclusions
Ranking of toxic potential based on the results of this intratracheal instillation test: Day 3: NM-105 = NM-104 > NM-103 >> Hombikat > NM-101 = PC105 > TIONA AT-1 = vehicle control Ranking of toxic potential based on the results of this intratracheal instillation test: Day 27: NM-105 > NM-104 > NM-103 >> Hombikat > NM-101 = PC105 = TIONA AT-1 = vehicle control Full recovery for Hombikat UV 100, NM-101, PC105 and TIONA AT-1 after 27 days

Overall remarks, attachments

7.2.3 Acute toxicity: dermal

7.2.4 Acute toxicity: other routes

7.3 Irritation / corrosion

7.4 Sensitisation

7.5 Repeated dose toxicity
7.6 Genetic toxicity

7.6.1 Genetic toxicity in vitro

Summary of the HH literature data status 03rd April 2014: Summary of Genotoxicity in vitro

<table>
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<th>Reference</th>
<th>Material/Size</th>
<th>Test Organism (Strain)/Test System</th>
<th>Method</th>
<th>Exposure/dose</th>
<th>Main findings</th>
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</thead>
<tbody>
<tr>
<td>Gurr et al. 2005</td>
<td>a. HOMBIKAT UV 100 (10 nm, anatase)</td>
<td>BEAS-2B bronchial epithelial cell line (human)</td>
<td>1. comet assay (+ enzyme digestion) 2. Micronucleus test</td>
<td>10 µg/ml (24h)</td>
<td>Nanosized particles but not 200 nm particles positive in either test.</td>
</tr>
<tr>
<td></td>
<td>b. Millenium PC500 (20 nm, anatase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. Rutile (200 nm)</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td>d. Anatase (200 nm)</td>
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7.6.2 Genetic toxicity in vivo

Endpoint study record: Genetic toxicity in vivo_NM 101_COMET INSTILLATION by NANOGENOTOX

Administrative Data

Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS

Study result type experimental result Study period 2012

Data source

Reference

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<tr>
<th>Reference type</th>
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<td>V Fessard</td>
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<td>Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis</td>
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Data access

Owner: NANOGENOTOX
Data protection claimed
yes, but willing to share

Cross-reference to same study
Genetic toxicity in vivo_NM101_MN Bone marrow instillation

Materials and methods
Type of genotoxicity
DNA damage and/or repair

Type of study
single cell gel/comet assay in rodents for detection of DNA damage

Test materials
Test material equivalent to submission substance identity
yes

Reference Material/Nanomaterial and Sample identification number
Identifier Reference Material/Nanomaterial
Identity NM 101

Test material identity
Identifier CAS number
Identity 13463-67-7
Identifier EC number
Identity 236-675-5

Test animals
Species
rat

Strain
Sprague-Dawley

Sex
male

Administration / exposure
Route of administration
intratracheal

Vehicle(s)
Rat Serum Albumin (RSA) 0.05% diluted (9:1 v/v) in 10x Phosphate buffer pH 7.4

Duration of treatment / exposure
3 administrations in a row: 1 administration at 0, 24 and 45 h Sampling time: 48 h
Doses / concentrations
1.15, 2.3, 4.6 mg/kg bw/d
Basis nominal conc.
No. of animals per sex per dose
5
Positive control(s)
Methyl MethaneSulfonate 25 mg/kg bw/d

Examinations
Tissues and cell types examined
lung, BAL fluid, liver, spleen, kidney
Evaluation criteria
Median % Tail intensity from >100 cells per organ
Statistics
Kruskall wallis one-way test for negative vs treated

Overall remarks, attachments
Attached full study report

Used Protocols: attached files

Attached document D2_WP4_ SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks Dispersion protocol
Remarks Data in the report and detailes porocol in annex

Applicant's summary and conclusion
Interpretation of results
negative
Conclusions
TiO2 NM-101 is not genotoxic in rats at the tested doses following a short-term exposition via intratracheal instillation with the comet assay

Cross-reference to other study

Endpoint study record: Genetic toxicity in vivo_NM 101_MN Bone marrow instillation by NANOGENOTOX

Administrative Data
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result Study period 2012
Data source

Reference

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<tr>
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Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Cross-reference to same study

NGTX_gentox_invivo_NM101_COMET Instillation_NRCWE

 Materials and methods

Type of genotoxicity

other: Cytogenetic damage

Type of study

micronucleus assay

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 101

Test material identity

Identifier CAS number
Identity 13463-67-7
Identifier EC number
Identity 236-675-5

Test animals

Species rat
Strain
Sprague-Dawley

Sex
male

Administration / exposure

Route of administration
intratracheal

Duration of treatment / exposure
3 administrations in a row: 1st at 0, 2nd at 24h and the 3rd at 45 h Sampling time: 48 h

Doses / concentrations
1.15, 2.3, 4.6 mg/kg bw/d

Basis nominal conc.

No. of animals per sex per dose
5

Positive control(s)
Methyl MethaneSulfonate 25 mg/kg

Examinations
Tissues and cell types examined
Bone marrow

Evaluation criteria
2000 immature erythrocytes per rat

Overall remarks, attachments

Attached full study report

Used Protocols: attached files

Attached document D2_WP4_ SOPs report: ENV/JM/MONO(2015)17/ANN1

Remarks Dispersion protocol


Remarks Data in the report and details protocol in annex

Applicant's summary and conclusion

Interpretation of results
negative

Conclusions
TiO2 NM-101 does not induce aneugenic/clastogenic damage in rats at the tested doses following a short-term exposition via intratracheal instillation with the micronucleus assay in bone marrow
Cross-reference to other study

7.7 Carcinogenicity

7.8 Toxicity to reproduction

7.9 Specific investigations

7.10 Exposure related observations in humans

7.11 Toxic effects on livestock and pets

7.12 Additional toxicological information

7.13 In vitro toxicological information

_Endpoint study record: cyto-toxicity by INIA_

**Administrative Data**

**Purpose flag** ( ) robust study summary ( ) used for classification ( ) used for MSDS

**Study result type** experimental result

**Data source**

**Data access**
other: performed and provided by INIA, Spain

**Materials and methods**

**Type of information**
An array of in vitro cytotoxicity assays were performed in a mammalian (H4IIE) and fish cell line (RTG-2) following exposure to NM 101 nanoparticles.

**Principles of method if other than guideline**
(MTT) Methyl thiazol tetrazolium salt reduction assay (NRU) Neutral red uptake assay (LDH) Lactate dehydrogenase

**Describe the scientific and technical basis of the test method**

**What biological/cellular model is the method based on?**
Rat hepatoma cell line (H4IIE) and Rainbow trout (Oncorhyncus mykiss) gonadal cell line (RTG 2)

**What biological endpoints/responses does this method address?**
cyto-toxicity
What methods/techniques are used for endpoints/responses determination?

Sample administration: 96-well cell culture plate. Exposure route: Directly on cells. Exposure duration: 24 h and 72 h. Concentration tested: 100-0.003 µg/mL.

Performance assessment of the method

Test materials

Details on test material
NM 101

Sample preparation/conditioning protocol

Appropriate amount of TiO2 particles in a 20 ml vial. Particles were transferred to exposure medium and vial washed thoroughly. Suspension were stirred overnight at approx. 900 rpm with a magnetic stirrer. Exposure dilutions were made during stirring. Unused suspensions were kept in closed bottles wrapped in aluminium foil and stirred for 1 h before reusing.

Method

Any other information on materials and methods incl. tables

Particle exposures: In a 96 well-plate, 100 µl of cell suspension at 2.5×10^5 cells ml−1 for the H4IIE cell line and 1×10^5 cells ml−1 for the RTG-2 cell line were added to each well and then incubated for 24 h at 37 °C and 5% CO2 for the H4IIE or 20°C and 5% CO2 for the RTG-2 cell line respectively. After 24 h, cells were exposed to the respective TiO2 nanoparticles with the highest nominal exposure concentration of 100 µg / mL. The remaining concentrations, ranging from 50 µg / mL to 0.003 µg /mL were produced by a 50 % downwards dilution for all assays. All treatments were done in triplicate. A positive control containing sodium dodecyl sulfate (SDS) was also included, ranging from 10 µg /mL to 0.3 µg /mL. The exposure duration was 24 h or 72 h. For 72 h exposures the cell media was renewed prior to exposure.

Cytotoxicity assays: MTT assay

Cell viability was determined via the ability of living cells to reduce the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described by (Mosmann, 1983). The cells were washed with PBS after the exposure to the respective particle and 100 µL of EMEM media without phenolred containing 0.5 mg /mL of MTT was added in each well. The plates were then incubated at the appropriate temperature and 5% CO2 for 4 h. The precipitated blue formazan product was then extracted using isopropanol as a solvent. The optical density (OD) of each well was determined at a wavelength of 570 nm using a plate reader (Tecan GENios, Madrid, Spain). The data was then expressed as a percentage of the control.

Neutral red uptake assay (NRU)

Cell viability was determined via the ability of intact lysosomes to retain the dye neutral red in living cells as described by (Borenfreund and Puerner, 1985). The cells were washed with PBS after the exposure to the respective particle and 100 µL of EMEM media without phenolred containing 0.05 mg /mL of neutral red was added in each well. The plates were then incubated at the appropriate temperature and 5% CO2 for 4 h. After incubation the cells were washed again with PBS and the neutral red retained by the cells was extracted using 100 µL of 1% acetic acid in 50% ethanol per well. The optical density (OD) of each well was determined at a wavelength of 550 nm using a plate reader (Tecan GENios, Madrid, Spain). The data was then expressed as a percentage of the control.

The lactate dehydrogenase (LDH) assay

The concentration of LDH in cells or released to the medium was determined as described by Brown et al., 2001. The assay is based on the ability of LDH to catalyze the conversion of pyruvate to lactate with simultaneous conversion of NADH to NAD+. The pyruvate not converted by LDH attaches to 2,4-dinitrophenylhydrazine and forms a brown complex. The intensity and therefore the measured absorption is inversely proportional to the LDH concentration. Triton X-100 is used as a positive control since its assumed to cause 100 % cell death and therefore represents the total releasable LDH.
Results and discussions
Remarks on results including tables and figures
The assays were conducted with H4IIE and RTG 2 cell lines with various concentrations and 24 h or 72 h as exposure durations. The MTT, NRU as well as the LDH assay, for assessing cytotoxicity, showed no negative effect due to exposure to the tested Titanium dioxide particles at the concentrations used (100 mg/L – 0,003 mg/L).

Applicant's summary and conclusion
Cross-reference to other study

Endpoint study record: cyto-toxicity by University of Graz
Administrative Data
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Data source
Data access
other: performed and provided by University of Graz, Austria
Cross-reference to same study
Materials and methods
Principles of method if other than guideline

Describe the scientific and technical basis of the test method
What biological/cellular model is the method based on?
buccal squamous epithelial TR 146 cells
What biological endpoints/responses does this method address?
cytotoxicity

What methods/techniques are used for endpoints/responses determination?
Formazan bioreduction: In order to examine cell viability, a CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used according to the instruction given by the manufacturer. 2 x 104 cells/200 µl medium were seeded in 96 well plates and cultured for 24 h. Subsequently, the medium was replaced by particles/serum-free medium dispersion in different concentrations and incubated for 4h and 24h. 20 µl of a MTS/PMS solution per well was added and re-suspended. After an incubation time of 4 h, the absorbance was measured at 490 nm with a VIS-plate reader (FLUOstar Optima, BMG, Labortechnik).
LDH release: To evaluate the lactate dehydrogenase (LDH) release, 2 x 104 cells/200 µl medium were seeded in a 96 well plate and incubated for 24 h. The medium was replaced by a particles/serum-free medium and incubated for 4h and 24h. LDH leakage was determined using a CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer’s instruction. Control wells (100% LDH release) were treated with 2 µl of lysis solution. 25 µl of the supernatant were mixed with 25 µl of the CytoTox-ONE Reagent in a white microtiter plate. At the end of 10 min incubation time (at RT), reaction was stopped by adding 12.5 µl stop solution. The fluorescence was recorded by fluorometer (FLUOstar Optima, BMG, Labortechnik) at 560 nm excitation wavelength and 590 nm emission wavelength. Number of replica: n=6 Frequency of Dosing: - Positive and negative control groups and treatment: untreated cell as negative control Solvent: serum-free medium Description of follow up repeat study: same conditions Criteria for evaluating results: negative control, cell viability, seeding cell density (calculated from the growth curve/prolifertation curve)

Describe the Standard Operating Procedure (SOP)
SOP description as a template
Sample administration: Particles dispersed in serum-free medium (n=6) •Exposure route: oral/buccal •Exposure duration: 4 and 24 h. •Concentration tested 1, 5, 10, 20, 50, 80, 100, 150, 200 µg/ml, (n=6)

Performance assessment of the method
Test materials

Sample preparation/conditioning protocol
Particles dispersed in serum-free medium

Results and discussions
Remarks on results including tables and figures
The cytotoxic effects of NM101 were assessed by a MTS assay after 4 and 24h. The data obtained from the particles showed negligible reduced mitochondrial activity/viability, indicating no cytotoxic effects. After 4 and 24h incubation time, more than 90 % viability was maintained. The membrane integrity was assessed by LDH release. NM101 particles displayed no significant influence on the membrane integrity independent on the concentration within 24h. The results of the MTS- and LDH-tests are listed in table 1 and 2 and illustrated in figure 5 and 6
Overall remarks, attachments

Overall remarks
Generally, nanoparticles are effective disrupters of cell plasma membranes (Lerouei et al.). There are two common types of disruption, i) nanoscale hole formation and ii) membrane thinning effects. A disruption of the membrane correlates with the enzyme leakage, dye diffusion, cytotoxicity and in-vitro particle uptake. Therefore, LDH and MTS investigations were performed. The results indicated that no membrane disruption occurred. These data could be confirmed by the viability assays.

Attached background material

![Figure 5](image5.png)

*Figure 5. Viability assay (MTS-test) of TR 146 cells treated with NM101 particles (4h and 24 h incubation time).*

![Figure 6](image6.png)

*Figure 6 Membrane integrity assay (LDH-test) of TR 146 cells treated with NM101 particles (4h and 24 h incubation time).*
Table 1 Results of the MTS-test of NM101

<table>
<thead>
<tr>
<th>Concentration [µg/ml]</th>
<th>Viability [%] after 4 h</th>
<th>Standard deviation [%]</th>
<th>Viability [%] after 24 h</th>
<th>Standard deviation [%]</th>
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Table 2 Results of the LDH-test of NM101

<table>
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<th>Concentration [µg/ml]</th>
<th>LDH release [%] after 4 h</th>
<th>Standard deviation [%]</th>
<th>LDH release [%] after 24 h</th>
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Applicant's summary and conclusion

Conclusions
NM101 particles, tested in concentrations up to 200 µg/ml, do not affect the viability and the membrane integrity of human buccal epithel cells under in-vitro conditions (manuscript in preparation).

Cross-reference to other study

Endpoint study record: buccal mucosa permeability by University of Graz

Administrative Data
Purpose flag ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Materials and methods

Principles of method if other than guideline


What biological/cellular model is the method based on?

excised porcine buccal mucosa

What methods/techniques are used for endpoints/responses determination?

Permeability studies:  • Exposure route: oral/buccal  • Exposure duration: 4 h  • Concentration tested: 100 µg/ml PBS (pH 7.4)  • Description of the method and give justification:, "no guidelines available" Porcine mucosa is the most similar one to the human mucosa in ultra structure as well as in enzyme activity and was obtained from freshly sacrificed pigs (age: < 6 months; Karneta Slaughter House, Graz, Austria). Ten minutes after slaughtering, the mucosa was immediately stored in 4°C Krebs buffer (KB), transferred to the laboratory and used within 1 hour post mortem. The underlying tissue was removed with a scalpel blade and carefully trimmed with surgical scissors to achieve uniform thickness. During preparation the tissue was rinsed with 4°C KB every two minutes to prevent dehydration of the tissue. Prior to every experiment, MTT-tests were carried out to assure the viability of the tissue. As negative control, samples were boiled in water for one hour to deactivate the tissue (zero value). The measured values were calculated as absorbance units per mg tissue (i.e., tetrazolinium reductase index (TR Index)). Additionally, the integrity of the tissue was checked. The integrity test of the membrane was carried out using methylene blue/ PBS (1mg/ml) and methylene blue/ EDTA/ PBS (1mg/0.5mM/ml).The oral barrier studies were performed with static Franz diffusion cells (PermeGear, USA, 11.28 mm jacketed cell with a flat ground (ground o-ring) joint and clear glass with an 8 ml receptor volume). Each cell consisted of a donor and of a receiver compartment. The receiver compartment was surrounded by a water jacket to assure a physiological temperature of 37 ± 0.5 °C throughout the experiment. The receiver compartment was filled with 7.8 ml PBS buffer and heated to 37 °C before use. A magnetic stirrer was used with an agitation of 300 rpm to assure equal distribution. Between the compartments the excised viable and integral sheet of mucosa was inserted and fixed with retainer clips in such a way that the epithelium faces the donor and the connective tissue region faces the receiver compartment. After an equilibration time of 30 min, the buffer in the donor compartment was replaced by TiO2 particles dispersed in PBS in a concentration of 100 µg/ml. After 4 h test duration, the mucosa was washed 3 times with PBS, fixed and embedded. Observation of the tissue samples was carried out by transmission electron microscopy and the particles were verified by element-analyses.  • Analytics (analytical verification) The penetration behavior of NM100 particles was evaluated by Transmission Electron Microscopy (TEM). The tissue was fixed
with 0.1M sodium phosphate buffered 2.5% glutaraldehyde overnight at 4°C and post-fixed in 1.0% osmium tetroxide. Dehydration was carried out through a graded series of ethanol to 100%. Subsequently, the tissue was transferred into propylene oxide and embedded into epoxy resin. Thin tissue sections were cut with a diamond knife and placed onto 300 mesh copper grids. The grids were not stained with heavy metals to prevent staining precipitates. Transmission Electron Microscopy images were obtained using a TEM model Tecnai equipped with an energy filter. Permeability studies: • Exposure route: oral/buccal • Exposure duration: 4 h • Concentration tested: 100 µg/ml PBS (pH 7.4) • Description of the method and give justification: "no guidelines available" Porcine mucosa is the most similar one to the human mucosa in ultra structure as well as in enzyme activity and was obtained from freshly sacrificed pigs (age: < 6 months; Karneta Slaughter House, Graz, Austria). Ten minutes after slaughtering, the mucosa was immediately stored in 4°C Krebs buffer (KB), transferred to the laboratory and used within 1 hour post mortem. The underlying tissue was removed with a scalpel blade and carefully trimmed with surgical scissors to achieve uniform thickness. During preparation the tissue was rinsed with 4°C KB every two minutes to prevent dehydration of the tissue. Prior to every experiment, MTT-tests were carried out to assure the viability of the tissue. As negative control, samples were boiled in water for one hour to deactivate the tissue (zero value). The measured values were calculated as absorbance units per mg tissue (i.e., tetrazolinium reductase index (TR Index)). Additionally, the integrity of the tissue was checked. The integrity test of the membrane was carried out using methylene blue/ PBS (1mg/ml) and methylene blue/EDTA/ PBS (1mg/0.5mM/ml). The oral barrier studies were performed with static Franz diffusion cells (PermeGear, USA, 11.28 mm jacketed cell with a flat ground (ground o-ring) joint and clear glass with an 8 ml receptor volume). Each cell consisted of a donor and of a receiver compartment. The receiver compartment was surrounded by a water jacket to assure a physiological temperature of 37 ± 0.5 °C throughout the experiment. The receiver compartment was filled with 7.8 ml PBS buffer and heated to 37 °C before use. A magnetic stirrer was used with an agitation of 300 rpm to assure equal distribution. Between the compartments the excised viable and integral sheet of mucosa was inserted and fixed with retainer clips in such a way that the epithelium faces the donor and the connective tissue region faces the receiver compartment. After an equilibration time of 30 min, the buffer in the donor compartment was replaced by TiO2 particles dispersed in PBS in a concentration of 100 µg/ml. After 4 h test duration, the mucosa was washed 3 times with PBS, fixed and embedded. Observation of the tissue samples was carried out by transmission electron microscopy and the particles were verified by element-analyses. • Analytics (analytical verification) The penetration behavior of NM100 particles was evaluated by Transmission Electron Microscopy (TEM). The tissue was fixed with 0.1M sodium phosphate buffered 2.5% glutaraldehyde overnight at 4°C and post-fixed in 1.0% osmium tetroxide. Dehydration was carried out through a graded series of ethanol to 100%. Subsequently, the tissue was transferred into propylene oxide and embedded into epoxy resin. Thin tissue sections were cut with a diamond knife and placed onto 300 mesh copper grids. The grids were not stained with heavy metals to prevent staining precipitates. Transmission Electron Microscopy images were obtained using a TEM model Tecnai equipped with an energy filter.

**Performance assessment of the method**

**Test materials**

**Details on test material**

in media: Zeta-Potential: -28.7mV agglomeration size: mean diameter 1292nm (PDI 0.168) RB adsorption constant: 0.02 ml/mg

**Sample preparation/conditioning protocol**

Particles dispersed in PBS and ultra-sonicated for 12h: mean diameter: 705.6 nm (PCS). Particle dispersion with a concentration of 0.4 mg/ml was prepared to determine the average particle size and the zeta potential. The particles were suspended in different physiological phosphate buffered saline. Ultra-
sonication was carried out for 12 h to ensure a high particle distribution. The hydrodynamic size and zeta potential of the particles were measured by photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments) at a detection angle of 173°. The surface hydrophobicity was determined via the Rose Bengal (RB) adsorption method. The adsorbed amount of the hydrophobic dye Rose Bengal (Sigma Aldrich, Vienna, Austria) onto the particle surface was measured (Müller et al.). The particles dispersed in PBS were incubated at different RB concentrations (10-50 µg/ml) for 3 hours at room temperature. After centrifugation (3 hours at 14,000 rpm), the free amount of RB in the supernatant was measured spectro-photometrically at 544 nm (FLUOstar Optima, BMG Labortechnik). The maximal amount bound was determined using a Scatchard Plot. Thereby, the binding constant was calculated according as: $r/a = KN - Kr$ where $N$ is the maximum amount bound (µg/mg), $r/a$ is the adsorbed amount of RB (µg/mg) per equilibrium concentration of RB (µg/ml) and $K$ is the binding constant (ml/µg).

**Results and discussions**

**Remarks on results including tables and figures**

The permeability of the NM101 across the buccal mucosa with a thickness of approximately 700 µm was determined. As illustrated in Figure 1, particles permeated the mucus layer and penetrated into the epithelium. It was found that particles were internalized by the cells of the superficial epithelium. However, NM101 particles were not detected in the basal lamina, the deepest part of the buccal epithelium. The existence of all particles was evaluated/confirmed by energy filtered TEM and verified by elemental mapping. The results of the permeability studies demonstrated that TiO2 particles (NM101) can permeate the mucus layer and penetrate into the stratum superficiale of the epithelium. However, they cannot reach deeper epithelial parts like the basal lamina. In previous studies we demonstrated that the buccal uptake is a function of the surface charge, the size and hydrophilicity/hydrophobicity. Concerning the physicochemical properties of NM 101, surface charge (zeta-potential: -28.7 mV dispersed in PBS) and hydrophilicity/hydrophobicity (0.02 µg/ml dispersed in PBS) would offer a good prerequisite for an optimal uptake into the buccal mucosa. However, it seems that the primary size of 7 nm do not fulfill the requirements for an efficient buccal uptake. This phenomenon could be based on the special epithelial surface of the buccal mucosa, which is covered by ridge-like folds, so called microplicae. We hypothesized that these microplicae might enhance the effect of size-dependent particle uptake based on thermodynamic driving forces (and diffusion kinetics). In previous studies it could be demonstrated that 200 nm positive and 200 nm neutral polystyrene nanoparticles were able to permeate the mucus layer readily and to penetrate the buccal epithelium to a high extent. 25 nm neutral particles also penetrated the mucosal tissue, however, with lower uptake efficiency and diffusion velocity than the larger ones. It seems that the mucus layer together with the buccal epithelium acts as a stronger barrier for smaller particles than for 200 nm particles [1,2].

**Overall remarks, attachments**

**Attached background material**
Applicant's summary and conclusion

Conclusions
The results of the permeability studies demonstrated that TiO2 particles can permeate the mucus layer and penetrate into the stratum superficiale of the epithelium.

Cross-reference to other study

8. ANALYTICAL METHODS

9. RESIDUES IN FOOD AND FEEDINGSTUFFS

10. EFFECTIVENESS AGAINST TARGET ORGANISMS

11. GUIDANCE ON SAFE USE