ENVIRONMENT DIRECTORATE 
JOINT MEETING OF THE CHEMICALS COMMITTEE AND 
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY 

DOSSIER ON TITANIUM DIOXIDE 
- PART 5 - NM 103 

Series on the Safety of Manufactured Nanomaterials 
No. 54 

This document is only available in PDF format.

JT03383138 
Complete document available on OLIS in its original format 
This document and any map included herein are without prejudice to the status of or sovereignty over any territory, to the delimitation of international frontiers and boundaries and to the name of any territory, city or area.
DOSSIER ON TITANIUM DIOXIDE
- PART 5 - NM 103
Dossiers also published in the Series on the Safety of Manufactured Nanomaterials:

No. 44, Dossier on Gold nanoparticles (2015)
No. 45, Dossier on Cerium oxide (2015)
No. 46, Dossier on Dendrimers (2015)
No. 47, Dossier on Nanoclays (2015)
No. 48, Dossier on Fullerenes (2015)
No. 49, Dossier on Multiwalled Carbon Nanotubes (MWCNTs) (2015)
No. 50, Dossier on Single-walled Carbon Nanotubes (SWCNTs) (2015)
No. 51, Dossier on Silicon dioxide (2015)
No. 52, Dossier on Zinc oxide (2015)
No. 53, Dossier on Silver nanoparticles (2015)

© OECD 2015
Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, RIGHTS@oecd.org, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France
ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 34 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD’s work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD’s workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials. More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD’s World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.
This publication is available electronically, at no charge.

For this and many other Environment, Health and Safety publications, consult the OECD’s World Wide Web site (www.oecd.org/chemicalsafety/)

or contact:

OECD Environment Directorate, Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org
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 guidelines1 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 NMs 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 testing2. 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 addressed3 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

---
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/oecdguidelinesforthetestingofchemicals.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.
[ENV/JM/MONO(2009)20/REV]⁴. The objective of this Guidance Manual was to guide sponsors⁵ 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 format⁶ 7. 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 end points 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

---

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)
the exploratory nature of the work would require subsequent follow-up work for example to review the 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\textsubscript{2}) manufactured nanomaterials which was prepared under the leadership of France and Germany. TiO\textsubscript{2} has been tested for a number of endpoints for: i) Nanomaterials Information / Identification; ii) Physical-Chemical Properties; iii) Environmental Fate; iv) Environmental Toxicology; v) Mammalian Toxicology; and vi) Material Safety. The data is presented in an IUCLID\textsuperscript{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)\textsuperscript{9}, or not.

France and Germany led the Testing Programme on nano-TiO\textsubscript{2}. This included the determination of data from the tests already completed using nano-TiO\textsubscript{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\textsuperscript{®} P 25 (P25) was chosen as \textit{principle material} meaning that all the relevant endpoints have been addressed for this material.

- Aeroxide\textsuperscript{®} 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\textsubscript{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:

\textsuperscript{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.

\textsuperscript{9} http://www.oecd.org/env/testguidelines
- **PC105** (JRC no. NM102)
  - provided by Cristal Global\(^{10}\) and delivered by EC/JRC, Lot-Nr.: 6292000312
- **Hombikat UV 100** (Sachtleben) identified as **NM-101** Titanium Dioxide
  - provided and delivered by EC/JRC, Lot-Nr.: 10780048
- **UV TITAN M212** (Sachtleben) (JRC no. NM104)
  - provided and delivered by EC/JRC, Lot-Nr.: 808001
- **UV TITAN M262** (Sachtleben) (JRC no. NM103)
  - provided by EC/JRC, Lot-Nr.: 933002
- **Tiona AT-1** (non-nano reference) (JRC no. NM100)
  - provided by Cristal Global\(^{11}\) and delivered EC/JRC, Lot-Nr.: 6111007957

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.

\(^{10}\) 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.

---

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 Laboratoris
- 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 metrlogie et d''Essais, Laboratoire National de metrlogie 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.
TABLE OF CONTENTS

PREAMBLE............................................................................................................................................. 7
FOREWORD.................................................................................................................................................. 10
ACKNOWLEDGMENTS.................................................................................................................................. 13
1. GENERAL INFORMATION .................................................................................................................. 19
   1.1 Identification....................................................................................................................................... 19
   1.2 Composition....................................................................................................................................... 19
   1.3 Identifiers .......................................................................................................................................... 19
   1.4 Analytical information ..................................................................................................................... 19
   1.5 Joint submission ................................................................................................................................. 19
   1.6 Sponsors ........................................................................................................................................... 19
   1.7 Suppliers ........................................................................................................................................... 19
   1.8 Recipients .......................................................................................................................................... 19
   1.9 Product and process oriented research and development ............................................................... 19
2. CLASSIFICATION AND LABELLING .............................................................................................. 20
   2.1 GHS ................................................................................................................................................... 20
   2.2 DSD - DPD ....................................................................................................................................... 20
3. MANUFACTURE, USE AND EXPOSURE ......................................................................................... 20
   3.1 Technological process ...................................................................................................................... 20
   3.2 Estimated quantities .......................................................................................................................... 20
   3.3 Form in the supply chain ................................................................................................................. 20
   3.4 Identified uses and exposure scenarios ............................................................................................ 20
   3.5 Uses advised against ....................................................................................................................... 20
   3.6 Waste from production and use ....................................................................................................... 20
   3.7 Exposure estimates .......................................................................................................................... 20
   3.8 Biocidal information ....................................................................................................................... 20
   3.9 Application for authorisation of uses ............................................................................................... 20
4. PHYSICAL AND CHEMICAL PROPERTIES ..................................................................................... 20
   4.1 Appearance ....................................................................................................................................... 20
   4.2 Melting point ..................................................................................................................................... 20
   4.3 Boiling point ..................................................................................................................................... 20
   4.4 Density ............................................................................................................................................. 20
   4.5 Particle size, size distribution .......................................................................................................... 21
   4.6 Vapour pressure ............................................................................................................................... 34
   4.7 N-octanol-water partition coefficient ............................................................................................... 34
   4.8 Water solubility, hydrophilicity, dispersibility .................................................................................. 34
   4.9 Solubility in organic solvents / fat solubility .................................................................................... 34
   4.10 Surface tension ............................................................................................................................... 34
   4.11 Flash point...................................................................................................................................... 34
   4.12 Auto flammability ........................................................................................................................... 34
   4.13 Flammability ................................................................................................................................... 34
   4.14 Explosiveness ................................................................................................................................... 34
   4.15 Oxidising properties ....................................................................................................................... 34
   4.16 Oxidation reduction potential ........................................................................................................ 34
5. ENVIRONMENTAL FATE AND PATHWAYS ........................................... 110

5.1 Stability .................................................. 110

5.1.1 Phototransformation in air ........................................ 110

5.1.2 Hydrolysis .................................................. 110

5.1.3 Phototransformation in water ........................................ 110

5.1.4 Phototransformation in soil ........................................ 110

5.1.5 Preliminary: Dispersion stability in water ....................... 110

5.1.6 Preliminary: Abiotic degradability and fate ..................... 112

5.2 Biodegradation ............................................. 112

5.2.1 Biodegradation in water: screening tests ......................... 112

5.2.2 Biodegradation in water and sediment: simulation tests .......... 112

5.2.3 Biodegradation in soil ........................................ 112

5.2.4 Mode of degradation in actual use ................................ 112

5.3 Bioaccumulation ........................................... 113

5.4 Transport and distribution ..................................... 113

5.4.1 Adsorption / desorption ..................................... 113

5.4.2 Henry's Law constant ........................................ 117

5.4.3 Distribution modelling ........................................ 117

5.4.4 Other distribution data ...................................... 117

5.6 Other relevant information ....................................... 123

6. ECOTOXICOLOGICAL INFORMATION ........................................... 123

6.1 Aquatic toxicity ........................................... 123

6.1.1 Short-term toxicity to fish .................................... 123

6.1.2 Long-term toxicity to fish .................................... 128

6.1.3 Short-term toxicity to aquatic invertebrates ....................... 128

6.1.4 Long-term toxicity to aquatic invertebrates ....................... 130

6.1.5 Toxicity to aquatic algae and cyanobacteria ..................... 138

6.1.6 Toxicity to aquatic plants other than algae ...................... 138

6.1.7 Toxicity to microorganisms .................................. 138

6.1.8 Toxicity to other aquatic organisms ............................ 138
6.2 Sediment toxicity ................................................................. 141
6.3 Terrestrial toxicity............................................................... 141
   6.3.1 Toxicity to soil macroorganisms except arthropods .............. 141
   6.3.2 Toxicity to terrestrial arthropods .................................... 147
   6.3.3 Toxicity to terrestrial plants ......................................... 147
   6.3.4 Toxicity to soil microorganisms ..................................... 147
   6.3.5 Toxicity to birds ....................................................... 147
   6.3.6 Toxicity to other above-ground organisms .......................... 147
6.4 Biological effects monitoring ............................................. 147
6.5 Biotransformation and kinetics ........................................... 147
6.6 Additional ecotoxicological information ............................... 147

7. TOXICOLOGICAL INFORMATION ........................................... 147
   7.1 Toxicokinetics, metabolism and distribution ......................... 147
      7.1.1 Basic toxicokinetics .............................................. 147
   7.2 Acute Toxicity ............................................................. 154
      7.2.1 Acute toxicity: oral .............................................. 154
      7.2.2 Acute toxicity: inhalation ...................................... 154
      7.2.3 Acute toxicity: dermal ......................................... 156
      7.2.4 Acute toxicity: other routes ................................... 156
   7.3 Irritation / corrosion ................................................... 156
   7.4 Sensitisisation ............................................................ 156
   7.5 Repeated dose toxicity ................................................ 156
      7.5.1 Repeated dose toxicity: oral ................................... 156
      7.5.2 Repeated dose toxicity: inhalation .............................. 156
      7.5.3 Repeated dose toxicity: dermal ................................ 159
      7.5.4 Repeated dose toxicity: other routes ........................... 159
   7.6 Genetic toxicity .......................................................... 160
      7.6.1 Genetic toxicity in vitro ....................................... 160
      7.6.2 Genetic toxicity in vivo ....................................... 185
      7.6.3 Photogenotoxicity .............................................. 196
   7.7 Carcinogenicity ........................................................... 197
   7.8 Toxicity to reproduction ............................................... 197
   7.9 Specific investigations ................................................ 197
   7.10 Exposure related observations in humans ......................... 197
   7.11 Toxic effects on livestock and pets ................................ 197
   7.12 Additional toxicological information ............................... 197
   7.13 In vitro toxicological information .................................. 197

8. ANALYTICAL METHODS ...................................................... 199
9. RESIDUES IN FOOD AND FEEDINGSTUFFS ............................... 199
10. EFFECTIVENESS AGAINST TARGET ORGANISMS ........................ 199
11. GUIDANCE ON SAFE USE .................................................. 199
Substance: Titanium Dioxide (UV Titan 262; NM 103)

1. GENERAL INFORMATION

1.1 Identification

Substance identification
Chemical name  Titanium Dioxide (UV Titan 262; NM 103)
Substance identification
Chemical name  13463-67-7_NM-103 Titanium Dioxide
Reference substance
titanium dioxide / 13463-67-7

Type of substance
Composition  other: Existing Chemical
Origin  element

1.2 Composition

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

2.1 GHS

2.2 DSD - DPD

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

4.3 Boiling point

4.4 Density
4.5 Particle size, size distribution

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

**Administrative Data**

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

Study result type  experimental result

**Data source**

**Reference**

Reference type  study report

Author  Carmen Nickel, Bryan Hellack, Stefan Gartiser, Felicitas Flach, Andreas Schiwy, Hanna Maes, Andreas Schäffer, Stephan Gabsch, Michael Stintz, Lothar Erdinger, Thomas A.J. Kuhlbusch

Year  2011

Title  Fate and behaviour of TiO2 nanomaterials in the environment, influenced by their shape, size and surface area

**Testing laboratory**  IUTA e.V. Duisburg, Hydrotex GmbH, Freiburg, RWTH Aachen, TU Dresden, Universitätsklinikum Heidelberg, Universität Duisburg-Essen, Germany

**Report no.**  FKZ 3709 65 417

**Materials and methods**

**Methods**

TEM

**Used Protocols:**
UVTitan M262-250nm_2_19.5kx.jpg

UVTitanM262-100nm_3_66kx.jpg
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

25 nm

Remarks on results including tables and figures

The representative TEM pictures confirm the particle size provided by the supplier (20nm).

Date: 21.06.12

Figure: NM-103

NM 103 – UV Titan M626
TiO$_2$ NM5 UV Titan M262

<table>
<thead>
<tr>
<th>d (nm)</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>33</td>
<td>6</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>38</td>
<td>1</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>45</td>
<td>2</td>
</tr>
<tr>
<td>47</td>
<td>1</td>
</tr>
</tbody>
</table>

Moda 25 nm
Overall remarks, attachments
Attached background material
TEM NNM 103.doc

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

Data Author Karl Franzens, Dr. Eva Roblegg and Sandra Blass
Title Sponsorship Program: Titanium Dioxide Report
source
Data access
other: performed and provided by University of Graz

Materials and methods
Methods
TEM
Overall remarks, attachments

Attached background material
Uni Graz_Roblegg_TEM.docx

**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

Reference

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Key intrinsic physicochemical characteristics of NANOGENOTOX nanomaterials</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bibliographic source</th>
<th>Testing laboratory</th>
<th>Report no.</th>
<th>D4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NANOGENOTOX</td>
<td>CODA-CERVA (B), INRS (F), IMC-BAS (BG)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Owner company</th>
<th>Company study no.</th>
<th>Report date</th>
<th>2012-05-01</th>
</tr>
</thead>
</table>

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

o 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. o 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.] o 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. o The ‘Detection module’ of iTEM was used for threshold-based detection of the NM. o 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.o 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. (In addition to the micrograph related information, the intermediate and annotated images obtained during image analysis and the results and reports of these analyses were stored in the database, linked to the original micrograph.) 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 Spearman rank correlation test. Since these assumptions were not met, the non-parametric Kruskal-Wallis one way ANOVA was performed and data were compared pairwise with Dunn’s Method to determine the micrograph and sample effects, and to determine the effect of sonication on the number of particles per grid area. The normality of the distributions and the homogeneity of variances were met for the mean values of the median mean diameter. A one way analysis of variance (ANOVA) was performed and data were compared pairwise with the Tukey test. The measured parameters were classified by principle component analysis using the SAS statistical software (SAS Institute Inc., Cary, NC, USA). Descriptive statistics and histograms were calculated in Sigmaplot (Systat, Cosinus Computing, Drunen, The Netherlands).

**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 Vibracecell™ 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 whas adjusts in order to allows for the attachment of the negatively charged silica NM to the EM grid. Alcian blue pretreatment introduced positive charges on the surface of pioiloform- and carbon-coated grids that tend to have a negative or neutral charge. (authors handexpierience 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 pioiloform- 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  

**Attached document** D4.2_TEM_characterisation: ENV/JM/MONO(2015)17/ANN4  
**Remarks** Data in the report and details porocol 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

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Reference Material/Nanomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>NM-103</td>
</tr>
</tbody>
</table>

State of test material

other: fluffy powder

Confidential details on test material

Commercial name: UV TITAN M262 (Sachtleben)

Results and discussions

* 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>
</tr>
</thead>
<tbody>
<tr>
<td>NM-103</td>
<td>26 ± 10 (1317)</td>
<td>26 ± 6 (101)</td>
<td>23.7 ± 5.9**</td>
</tr>
</tbody>
</table>

* Manual measurement
** Manual measurement using ImageJ software.

Remarks on results including tables and figures
Figure 1 NM103: Selected TEM micrographs showing the range from coarse µm-size aggregates (A) to small nanosize (B) in the sample. C) Representative TEM-Micrograph illustrating well-dispersed titanium dioxide aggregates showing typical aggregate/agglomerate size of 100 to 200 nm. Individual single nanoparticles are also present. Primary particles are typically smaller than 20 nm along the shortest dimensions. Note the elongated morphology of several particles.

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 porocol in annex

Applicant's summary and conclusion

Conclusions

very good correspondence between AFM and TEM values

Cross-reference to other study

Endpoint study record: Size distribution and intensity averaged mean size of aggregates by DLS and SAXS by NANOGENOTOX

Administrative Data

Purpose flag ( ) 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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K A Jensen</td>
<td>2013</td>
</tr>
</tbody>
</table>

| Title          | D4.5: Surface charge, hydrodynamic size and size distributions by zetametry, dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) in optimized aqueous suspensions for titanium and silicon dioxide. |
| Bibliographic source | NANOGENOTOX |
| Testing laboratory | CEA (F) |
| Owner company | |
| Company study no. | |

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Cross-reference to same study

End point : Homogeneity. Description of the method, instrument and sample preparation.

Materials and methods

Methods

DLS and SAXS

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 porocol in annex

Data gathering

Reproducibility

3 measurements
Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM-103

State of test material

other: fluffy powder in dispersion

Confidential details on test material

Commercial name: UV TITAN M262 (Sachtleben)

Results and discussions

Table 0.2: Size parameters and standard deviations from DLS measurements averaged on a given number of TiO₂ samples prepared by ultrasonication (20 min - 40 % amplitude) in HNO₃ 10⁻² M. Z-average, polydispersity index, position and width of the main peak in intensity size distribution.

<table>
<thead>
<tr>
<th>TiO₂ nanomaterial (total number of samples)</th>
<th>Z-Average (nm)</th>
<th>PdI</th>
<th>Intensity distribution main peak (nm)</th>
<th>FWHM main peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM103 (6)</td>
<td>113.2 ± 3.2</td>
<td>0.242 ± 0.018</td>
<td>138.4 ± 7.7</td>
<td>73.6 ± 11.0</td>
</tr>
</tbody>
</table>

Remarks on results including tables and figures

Figure 0.2: DLS intensity size distributions (left) and number size distributions (right) for suspensions of TiO₂ nanomaterials dispersed by ultrasonication (20 min - 40 % amplitude) in HNO₃ 10⁻² M.

For NM103, NM104 and NM105, distributions are quite well centered at 100-150 nm with a thinner distribution for NM105.
Table 0.3: Structure and size parameters extracted from SAXS data fitting by the unified model from TiO₂ suspensions ultrasonicated (20 min - 40 %) in HNO₃ 10⁻² M. Gyration radius of primaries and aggregates (Rg₁ and Rg₂), fractal dimension Dᵢ and number of particles per aggregate.*NM102 cannot be perfectly fitted at low q with Df < 3.

<table>
<thead>
<tr>
<th>TiO₂ nanomaterial</th>
<th>2 Rg₁ (nm)</th>
<th>2 Rg₂ (nm)</th>
<th>Dᵢ</th>
<th>Npart/agg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM103</td>
<td>26</td>
<td>140</td>
<td>2.2</td>
<td>113</td>
</tr>
</tbody>
</table>

The structure and main size parameters determined by the model, *i.e.* radius of gyration of primary particles (Rg₁), radius of gyration of aggregates (Rg₂) fractal dimension (Dᵢ) and average number of primaries per aggregates (Npart/agg) are reported in Table 0.3. The full sets of parameters used for the fit of experimental curves with the unified model are gathered in appendix E.

**Figure 0.3:** SAXS diffractograms fitted by the unified model for TiO₂ suspensions ultrasonicated (20 min - 40 %) in HNO₃ 10⁻² M.*NM102 cannot be perfectly fitted at low q with Df < 3.

**Overall remarks, attachments**

**Attached full study report**

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

**Remarks**  Dispersion protocol

**Attached document**  D4.5_ZETA_DLS_SAXS_analysis: ENV/JM/MONO(2015)17/ANN6

**Remarks**  Data in the report and details porocol in annex

**Applicant's summary and conclusion**

**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/DTA by NANOGENOTOX

Administrative Data

<table>
<thead>
<tr>
<th>Purpose flag</th>
<th>key study (X) robust study summary ( ) used for classification ( ) used for MSDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study result type</td>
<td>experimental result</td>
</tr>
<tr>
<td>Study period</td>
<td>2013</td>
</tr>
</tbody>
</table>
Data access

other: owner: NANOGENOTOX

Materials and methods

Endpoint investigated

other: mass lost by TGA and DTA

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 combustable 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 aluina 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-103

Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

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

TGA is short for thermograviometric 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:

- Weigh container.
- Fill container with material. Do not stamp it, as this may affect the evaporation/decomposition temperature.
- 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 NM103, there is a small but gradual weight loss, which may in fact be due to evaporation/combustion in several steps. There appears to be a change in the slope around 200°C, but the measurement is noisy and it is difficult to be sure. However the weight loss is above 100°C and is most likely due to a coating. According to the DTA/TG results, there are no indications of any significant phase transformation.
Overall remarks, attachments

Attached full study report

Remarks: Dispersion protocol

Remarks: Data in the report and detailes porocol in annex

Applicant's summary and conclusion

Conclusions

Small but gradual weight loss, which may in fact be due to evaporation/combustion in several steps.
There appears to be a change in the slope around 200°C, but the measurement is noisy and it is difficult to be sure. However the weight loss is above 100°C and is most likely due to a coating. According to the DTA/TG results, there are no indications of any significant phase transformation.

Cross-reference to other study

**Endpoint study record: composition by EDS by NANOGENOTOX**

**Administrative Data**

<table>
<thead>
<tr>
<th>Purpose flag</th>
<th>key study (X) robust study summary ( ) used for classification ( ) used for MSDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study result type</td>
<td>experimental result</td>
</tr>
</tbody>
</table>

**Data source**

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

**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 a 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

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Reference Material/Nanomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>NM 103</td>
</tr>
</tbody>
</table>

Details on test material

Commercial name: UV TiTAN M262

Results and discussions

Results

Minor and trace elements were given in ppm to enable direct comparison with results reported for the ICP analysis.

NM103 contained 3.4 wt% Al and trace amounts of Si and S.

Remarks on results including tables and figures

Table 0-4 Elemental concentrations by EDS measurements on TiO₂ performed at IMC-BAS.

<table>
<thead>
<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>
</thead>
<tbody>
<tr>
<td>NM103</td>
<td>34300</td>
<td>6800</td>
<td>2600</td>
<td>54.74</td>
<td>600</td>
<td>40.82</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ppm by weight

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

Reference

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
<th>Title</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>
</tr>
</tbody>
</table>

Bibliographic source

<table>
<thead>
<tr>
<th>Testing laboratory</th>
<th>Report no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CODA-CERVA (B)</td>
<td>D4.3</td>
</tr>
</tbody>
</table>

Owner company

Company study no.

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, S, 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

Identifier: Reference Material/Nanomaterial
Identity: NM-103

Details on test material
Commercial name: UV TiTAN M262

Results and discussions

Results
Tables 5-8 and 5-9 show the elemental concentration ranges found after screening the TiO₂ samples by ICP-OES. The most abundant impurities (> 1 wt%) were found to be Al in NM103.

Table 5-5. Graphical summary table with the impurity ranges found in titanium dioxide.

![Graphical summary table]

Table 5-6. Overview of impurities detected in titanium dioxide NM.

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Vial ID n°</th>
<th>Impurities</th>
<th>Impurities</th>
<th>Impurities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt; 0.01%</td>
<td>0.005 – 0.01%</td>
<td>0.001 – 0.005%</td>
</tr>
<tr>
<td>NM-103</td>
<td>0584 &amp; 0585</td>
<td>Al (&gt;1%), Na, S</td>
<td>Ca</td>
<td>Fe, K, Mg, Zr</td>
</tr>
</tbody>
</table>

* Near 0.01%

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

Cross-reference to other study
4.24 Agglomeration/aggregation

**Endpoint study record: Agglomeration/aggregation by Institute of Energy and Environmental Technology (IUTA)**

**Administrative Data**

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

**Data source**

- **Reference type**: study report

**Author** Carmen Nickel, Bryan Hellack, Stefan Gartiser, Felicitas Flach, Andreas Schiwy, Hanna Maes, Andreas Schäffer, Stephan Gabsch, Michael Stintz, Lothar Erdinger, Thomas A.J. Kuhlbusch

**Year**: 2011

**Title**: Fate and behaviour of TiO2 nanomaterials in the environment, influenced by their shape, size and surface area

**Testing laboratory**: IUTA e.V. Duisburg, Hydrotech GmbH, Freiburg, RWTH Aachen, TU Dresden, Universitätsklinikum Heidelberg, Universität Duisburg-Essen, Germany

**Report no.**: FKZ 3709 65 417

**Materials and methods**

**Used Protocols**

Standard operating procedure – Preparing Titanium dioxide suspensions in deionised water

1. Aim of the SOP
2. Background
3. Preliminary results
4. Preparing suspension

1. **Aim of the Standard Operating Procedure (SOP)**
   The aim of this Standard Operating Procedure is the preparation of a stable nanoscale Titanium dioxide suspension for environmental testing within the Project 3709 65 417 and afford reproducible results in different laboratories (comprehensible proceedings). The SOP describes the proceedings which are suitable for preparing a stable TiO2 nanoparticle suspension in this project for P25, PC105 and UV Titan M262.

2. **Background Suspension Requirements**
   - The suspension must be stable at least for 24 h (a variance of 10% is accepted).
   - An appropriate stability of a suspension is declared as a constant particle size distribution, concentration and zeta potential.
   - Stability criteria:
     - Optical observation (no visible sedimentation of the particles)
     - Size of the particles in the suspension
     - Zeta potential
     - Particle concentration
     - pH value of the suspension
     - Conductance of the suspension

3. **Preliminary results**
   Preliminary results show that a sufficient stability is warranted if the suspension (100 mg TiO2 material / 100 mL deionised water in a 250 mL beaker glass) was sonicated for 10 minutes.

4. **Preparing suspension**
   - For preparing suspension deionised water was used (pH 5.0 - variance of 10%).
   - A defined amount of the nanomaterial - here 100 mg of the solid material was weighted in a 250 mL beaker glass (a variance of 1% is accepted).
   - After this 100 mL of deionised water was carefully added to the material.
   - The beaker glass with the nanoparticle suspension was sonicated with a Bandelin Sonoplus HD 2200 ultrasonic homogeniser for 10 minutes* with a pulse of 0.2 / 0.8.
   - The horn of the ultrasonic homogeniser was dipped into the suspension and placed in the
middle of the beaker glass with a distance between horn and bottom of the beaker glass of approximately 1 cm. - For sonication the beaker glass with the suspension was put in a bigger beaker glass with cold/ice water to minimize the heating of the suspension during the sonication. - After use the horn was cleaned with ethanol and afterwards with deionised water. - After sonication the suspension was characterised to its size distribution – using a DLS instrument. Note: the sonication time must be adapted to the volume of the prepared suspension, diameter of the beaker glass, the concentration of the nanoparticles and the rated power of the ultrasonic instrument.

**Data gathering**

**Instruments**

In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanoparticle in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter / Zeta Sizer ZS - Malvern Instruments; Nanophox – Sympatec, size only).

**Test materials**

**State of test material**

dispersion

**Results and discussions**

**Agglomerate/aggregate diameter**

**Mean diameter**

180 nm

**Remarks on results including tables and figures**

Average of DLS measurements (this study) after 10 min sonication; n=5. stable over a time period of 24 h

![Table 4: DLS measurements of P25, PC105 and UV Titan M262 suspension with a concentration of 100 mg/L in 100 mL after 10 min sonication (200 W homogeniser); n = 5.](image)
Figure 6: Z-average of 100 mg/L P25, PC105 and UV Titan M262 in 100 mL DI water, sonication for 10, 15, 20, 30 and 40 min with SD as error bars; n = 10.

Figure 7: pH dependent DLS measurements of the Z-average of P25, PC105 and UV Titan M262 at pH 5, 7, 9 and 10 in DI water with SD as error bars; n = 3.
Overall remarks, attachments

Attached background material
diagramme Dispersion.docx

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

Aggregation Index

Remarks on results including tables and figures

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. The measurements showed, that the size distribution in media is comparable to the size distribution in water (not measured for low concentration)
1- Particle size distribution in media suspensions

Graphic 1: Particle size distribution in water at t = 0h
Graphic 2: Particle size distribution at $t=0\text{h}$

Graphic 3: Particle size distribution at $t=24\text{h}$
Graphic 4: Particle size distribution at t = 72h

Overall remarks, attachments
Attached background material
diagramm agglomeration.doc

*Endpoint study record: Agglomeration/aggregation by by Institute of Energy and Environmental Technology (IUTA) (2)*

Administrative Data

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

Data source

Reference
Reference type study report
Author Carmen Nickel, Bryan Hellack, André Nogowski, Frank Babick, Michael Stintz, Hanna Maes, Andreas Schäffer, Thomas Kuhlbusch
Year 2013
Title Mobility, fate and behaviour of TiO2 nanomaterials in different environmental media
Testing laboratory IUTA e.V. Duisburg, TU Dresden, RWTH Aachen, Universität Duisburg-Essen, Germany
Report no. FKZ 3710 65 414

Materials and methods

Methods
DLS and micro-electrophoresis by using video microscopy
**Used Protocols**

Standard operating procedure – Preparing Titanium dioxide suspensions in deionised water

1. **Aim of the SOP**
2. **Background**
3. **Preliminary results**
4. **Preparing suspension**

**Aim of the Standard Operating Procedure (SOP)**

The aim of this Standard Operating Procedure is the preparation of a stable nanoscale Titanium dioxide suspension with minimal invasive methods to minimize surface degradation within the Project 3710 65 414 and afford reproducible results in different laboratories (comprehensible proceedings). The SOP describes the preparation steps for a stable TiO2 nanomaterial suspension in this project for UV Titan M262.

**2. Background**

**Suspension Requirements**

- The suspension must be stable at least for 24 h (a variance of 10% is accepted).
- An appropriate stability of a suspension is declared as a constant particle size distribution, concentration and zeta potential.
- Stability criteria
  - Optical observation (no visible sedimentation of the particles)
  - Size of the particles in the suspension
  - Zeta potential
  - Particle concentration
  - pH value of the suspension
  - Conductance of the suspension

**Necessary Instrumentation**

- A sensitive analytical balance.
- Sonication equipment with sufficient rated power.
- Sensitive instrument detecting the particle size distribution and the zeta potential in aqueous media.

**3. Preliminary results**

Preliminary results show that a sufficient stability is warranted if the suspension (10 mg TiO2 material / 100 mL deionised water in a 250 mL beaker glass) was sonicated for 2 min for 100 mL or 1 min for 50 mL and stirred for 60 min.

**4. Preparing suspension**

- For preparing suspension deionised water was used (pH 5.0 - variance of 10%).
- A defined amount of the nanomaterial - here 10 mg of the solid material was weighted in a 250 mL beaker glass (a variance of 1% is accepted).
- After this 100 mL of deionised water was carefully added to the material.
- The beaker glass with the nanomaterial suspension was sonicated with a Bandelin Sonoplus HD 2200 ultrasonic homogeniser for 2 minutes* with a pulse of 0.2 / 0.8.
- The horn of the ultrasonic homogeniser was dipped into the suspension and placed in the middle of the beaker glass with a distance between horn and bottom of the beaker glass of approximately 1 cm.
- For sonication the beaker glass with the suspension was put in a bigger beaker glass with cold/ice water to minimize the heating of the suspension during the sonication.
- Afterwards the suspension was stirred for 60 min using a magnetic stirrer.
- After sonication the suspension was characterised to its size distribution and zeta potential.

**Note:** the sonication time must be adapted to the volume of the prepared suspension, diameter of the beaker glass, the concentration of the nanomaterials and the rated power of the ultrasonic instrument.

**Data gathering**

**Instruments**

In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanomaterial in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter coupled with electrophoretic light scattering for the calculation of the zeta potential of the suspended particles) and micro-electrophoresis by using video microscopy.

**Test materials**

**State of test material**

dispersion
Any other information on materials and methods incl. tables
Suspension, particles were suspended in DI water with a pH of 5. The pH was stepwise adjusted using 0.1 M HCl and 0.1 M NaOH and the coating stability was analysed. As basic conditions DI water was used and the change of the zeta potential was detected as function of the pH and afterwards as function of ionic strength and dissolved organic carbon (DOC) content (0.01 M; 0.001 M; 0.0001 M CaCl2 solution and 2.5 mg/L, 5 mg/L and 10 mg/L DOC).

Results and discussions

Agglomerate/aggregate diameter

Mean diameter

245.5 nm

Aggregation Index

Mean

Standard deviation 5.78

Remarks on results including tables and figures

Average of Intensity based DLS measurements (this study) after 1min sonication and 60 min stirring; n = 3. The suspension was stable over a time period of 24 h at pH 5, close to the IEP agglomeration and sedimentation was visually detected, no DLS measurements could be conducted. The material lost his hydrophobic behaviour after it was wetted with water and after suspension preparation. By using the established SOP nearly all of the Dimethicon from the surface of the nanomaterials was released. On average 87 % were detected in the supernatant of the suspension by ICP MS. The IEP and ICP measurements indicate that the aluminium oxide coating was not significantly affected. The zeta potential measurements detected an IEP around pH 8 – 9 which is in accordance with the IEP of aluminium oxide (Shin et al. 2006; Kosmulski 2006) – (final report FKZ (UFOPPlan) 3710 65 414). With increasing ionic strength the IEP was shifted to more basic conditions. At the highest test concentration of CaCl2 no IEP was detected. Agglomeration of the suspension was visually detected. With addition of DOC for all tested concentration the zeta potential was shifted to a negative value for all tested pH values. No IEP can be detected. The zeta potential and agglomerate size in suspension was stable over the whole pH range. If both CaCl2 and DOC were added, the zeta potential was negative and the IEP was not detected.

Figure 6: Average size (xCum) and polydispersity index from dynamic light scattering measurements, when...
Overall remarks, attachments
Attached background material
Agglomeration NM103 NM104.docx

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 Author Karl Franzens, 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
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 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). 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
Aggregation Index
Remarks on results including tables and figures
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

Results of the particle characterization of NM103 in different biological media

<table>
<thead>
<tr>
<th>NM103</th>
<th>0.4 mg/ml TiO2 particles (NM103, 20 nm, hydrophobic rutile, Sachtleben M262)</th>
<th>untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Size (d.nm)</td>
<td>Z-Average (d.nm)</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>973,2</td>
<td>671,6</td>
</tr>
<tr>
<td>PBS</td>
<td>1977</td>
<td>1397</td>
</tr>
<tr>
<td>DMEM + L-Glutamine</td>
<td>2255</td>
<td>1665</td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>1040*/4593</td>
<td>828,8</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>991,1</td>
<td>653,2</td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>1156</td>
<td>683,3</td>
</tr>
</tbody>
</table>

<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>2649</td>
<td>1977</td>
<td>0,393</td>
<td>39,1</td>
<td>6,08</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>1629*/4619</td>
<td>2275</td>
<td>0,442</td>
<td>-20,8</td>
<td>16,5</td>
<td></td>
</tr>
<tr>
<td>DMEM + L-Glutamin</td>
<td>4043</td>
<td>3551</td>
<td>0,279</td>
<td>-8,44</td>
<td>25,5</td>
<td></td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>275,6*/434</td>
<td>4</td>
<td>263,5</td>
<td>0,243</td>
<td>-9,98</td>
<td>19,1</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>432,4*/488</td>
<td>1</td>
<td>345,8</td>
<td>0,25</td>
<td>-12</td>
<td>19,8</td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>370,9</td>
<td>286,9</td>
<td>0,196</td>
<td>-12,4</td>
<td>-11,9</td>
<td>25,6</td>
</tr>
</tbody>
</table>

<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>765,3*/504</td>
<td>1</td>
<td>596,9</td>
<td>0,393</td>
<td>39,1</td>
<td>6,08</td>
</tr>
<tr>
<td>PBS</td>
<td>1449*/5037</td>
<td>1</td>
<td>1350</td>
<td>0,25</td>
<td>-20,9</td>
<td>11,2</td>
</tr>
<tr>
<td>DMEM + L-Glutamin</td>
<td>2916</td>
<td>2268</td>
<td>0,264</td>
<td>-8,76</td>
<td>15,4</td>
<td></td>
</tr>
<tr>
<td>DMEM + 1%</td>
<td>684,1*/494</td>
<td>526,8</td>
<td>0,317</td>
<td>-10,0</td>
<td>15,4</td>
<td></td>
</tr>
</tbody>
</table>
Results of the particle characterization with a Mastersizer 2000

<table>
<thead>
<tr>
<th>Medium</th>
<th>Size(0.1)</th>
<th>Size(0.5)</th>
<th>Size(0.9)</th>
<th>Sonification period</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQ Wasser</td>
<td>17297</td>
<td>621053</td>
<td>1503706</td>
<td>0 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>446</td>
<td>2003</td>
<td>6811</td>
<td>1 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>530</td>
<td>3355</td>
<td>7986</td>
<td>2 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>689</td>
<td>4255</td>
<td>9195</td>
<td>3 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>1134</td>
<td>4681</td>
<td>9988</td>
<td>4 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>1808</td>
<td>5083</td>
<td>10590</td>
<td>5 min US</td>
</tr>
</tbody>
</table>

Overall remarks, attachments

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.

Attached background material

Uni Graz_Roblegg_Agglomeration NM103.docx

Endpoint study record: Agglomeration/aggregation by DLS and SAXS by NANOGENOTOX

Administrative Data

Purpose flag  ( ) 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>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>K A Jensen</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>D4.5: Surface charge, hydrodynamic size and size distributions by zetametry, dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) in optimized aqueous suspensions for titanium and silicon dioxide.</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>NANOGENOTOX</td>
</tr>
<tr>
<td>Testing laboratory</td>
<td>CEA (F)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D4.5</td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Cross-reference to same study

End point: Homogeneity. Description of the method, instrument and sample preparation.

Materials and methods

Methods

DLS and SAXS

Details on methods and data evaluation

All details and discussion are detailed in the document ANN4_D4.5_ZETA_DLS_SAXS_analysis. Dispersion protocol is available in: nanogenotox dispersion protocol.pdf / 777.29 KB (application/pdf): ENV/JM/MONO(2015)17/PART1/ANN1

Used Protocols: attached files

Attached document


Data gathering

Reproducibility

3 measurements

Test materials

Test material equivalent to submission substance identity

yes
Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM-103

State of test material
other: fluffy powder in dispersion

Confidential details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Results and discussions

Table 0.1: Size parameters and standard deviations from DLS measurements averaged on a given number of TiO$_2$ samples prepared by ultrasonication (20 min - 40 % amplitude) in HNO$_3$ 10$^{-2}$ M. Z-average, polydispersity index, position and width of the main peak in intensity size distribution.

<table>
<thead>
<tr>
<th>TiO$_2$ nanomaterial (total number of samples)</th>
<th>Z-Average (nm)</th>
<th>PdI</th>
<th>Intensity distribution main peak (nm)</th>
<th>FWHM main peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM103 (6)</td>
<td>113.2 ± 3.2</td>
<td>0.242 ± 0.018</td>
<td>138.4 ± 7.7</td>
<td>73.6 ± 11.0</td>
</tr>
</tbody>
</table>

Remarks on results including tables and figures

Figure 0.7: DLS intensity size distributions (left) and number size distributions (right) for suspensions of TiO$_2$ nanomaterials dispersed by ultrasonication (20 min - 40 % amplitude) in HNO$_3$ 10$^{-2}$ M.

For NM103, NM104 and NM105, distributions are quite well centered at 100-150 nm with a thinner distribution for NM105.
Table 0.2: Structure and size parameters extracted from SAXS data fitting by the unified model from TiO₂ suspensions ultrasonicated (20 min - 40 %) in HNO₃ 10⁻² M. Gyration radius of primaries and aggregates (Rg₁ and Rg₂), fractal dimension D_f and number of particles per aggregate.*NM102 cannot be perfectly fitted at low q with D_f < 3.

<table>
<thead>
<tr>
<th>TiO₂ nanomaterial</th>
<th>2 Rg₁ (nm)</th>
<th>2 Rg₂ (nm)</th>
<th>D_f</th>
<th>N_{part/agg}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM103</td>
<td>26</td>
<td>140</td>
<td>2.2</td>
<td>113</td>
</tr>
</tbody>
</table>

The structure and main size parameters determined by the model, i.e. radius of gyration of primary particles (Rg₁), radius of gyration of aggregates (Rg₂) fractal dimension (D_f) and average number of primaries per aggregates (N_{part/agg}) are reported in Table 0.3. The full sets of parameters used for the fit of experimental curves with the unified model are gathered in appendix E.

Figure 0.8: SAXS diffractograms fitted by the unified model for TiO₂ suspensions ultrasonicated (20 min - 40 %) in HNO₃ 10⁻² M.*NM102 cannot be perfectly fitted at low q with D_f < 3.

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 porocol in annex

Applicant's summary and conclusion

Cross-reference to other study

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>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>KA Jensen</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>NRCWE (DK) and IMC-BAS (BG), LNE</td>
</tr>
<tr>
<td>Company study no.</td>
<td>Report no.</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 a 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 gathering

Instruments

The data from NRCWE were measured at room temperature (25°C) on a Bruker D8 Advanced Diffractometer in reflection mode with Bragg-Brentano geometry. The analysis were made using CuKα1 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 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 2θ 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 using CuKα1 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 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 2θ 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 be documented for detailed data comparisons using e.g., a large crystallite standard. For the analysis, NRCWE used a CeO$_2$ (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 radian angle.

**Reproducibility**

**Test materials**

**Test material equivalent to submission substance identity**

eyes

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

**Identifier** Reference Material/Nanomaterial

**Identity** NM-103

**State of test material**

other: fluffy powder

**Confidential details on test material**

Commercial name: UV Titan M262

**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 TiO$_2$ 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 103 is rutile
Remarks on results including tables and figures

*Table 0-3* Crystallite sizes (nm) determined from measurements on NM103, Rutile.

<table>
<thead>
<tr>
<th>Vial</th>
<th>LNE Scherrer Equation</th>
<th>IMC-BAS Peak fit, FWHM vs standard</th>
<th>IMC-BAS Topas 4.2, standard less</th>
<th>Fullprof, quartz standard</th>
<th>Scherrer Equation*</th>
<th>NRCWE Topas 4.1, IB</th>
<th>Topas 4.1 FWHM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0223</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.9 ± 5.6</td>
<td>25.4 ± 1.8</td>
<td>28.4 ± 1.9</td>
</tr>
<tr>
<td>0541</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.4 ± 4.7</td>
<td>24.7 ± 1.7</td>
<td>27.5 ± 1.7</td>
</tr>
<tr>
<td>0547</td>
<td>17.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0615</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0617</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0618</td>
<td></td>
<td>19.42 ± 4.7 (1.03)</td>
<td>18.03 ± 1.8 (5.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2097</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.7 ± 4.5</td>
<td>25.0 ± 1.7</td>
<td>27.8 ± 1.7</td>
</tr>
</tbody>
</table>

* Based on reflections: 101, 200, 105, 211, 116 and 220; * LNE also only identified rutile in the sample.

*Table 0-4* Summary of XRD sizes calculated for TiO₂ 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>IMC-BAS Peak fit</td>
<td>57</td>
<td>5</td>
<td>18</td>
<td>19</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>IMC-BAS TOPAS</td>
<td>62</td>
<td>5</td>
<td>16</td>
<td>19</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>IMC-BAS Fullprof</td>
<td>168</td>
<td>7</td>
<td>18</td>
<td>20</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>NRCWE Scherrer eq.</td>
<td>&gt; 100</td>
<td>7</td>
<td>23</td>
<td>26</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>NRCWE TOPAS, IB</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, 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>

* Size-data not reliable due to large crystallite size.

**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 porocol 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, Spain

Materials and methods

Methods
BET

Results and discussions

Specific surface area
Mean  54 m²/g
Standard deviation

Remarks on results including tables and figures
The surface area measured by BET (Table 3). The measured values are lower but very close to the information provided by the supplier.
Date: 21.06.12

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
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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>KA Jensen</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 4.4: Determination of specific surface area of NANOGENOTOX nanomaterials</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>CEA (F)</td>
</tr>
<tr>
<td>Testing laboratory</td>
<td>Report no. D4.4</td>
</tr>
<tr>
<td>Owner company</td>
<td>Company study no.</td>
</tr>
<tr>
<td>Report date</td>
<td></td>
</tr>
</tbody>
</table>
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 1mm 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 3600s, 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 physic-chemical properties of materials. This procedure was applied in the framework of NANOGENOTOX among others to characterize SiO2 manufactured nanostructures as raw powders and SiO2 in aqueous suspensions.

### Used Protocols: attached files

- **Attached document**
  - SOP_SAXS_CEA.doc / 2.38 MB (application/msword):

### 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 (λ= 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.

#### 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-103

#### State of test material

- other: fluffy powder

#### Confidential details on test material

- Commercial name: UV Titan M262
Results and discussions
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 NM103. $I(q)$ representations on the left; $I(q)q^4$ representation revealing Porod’s plateaus on the right.

It should be noted that each NM displays a specific curve, revealing different nanostructures of those materials (except for NM103 and NM104 which are morphologically identical and differ only from their organic coating). Therefore, it is possible to discriminate between nanomaterials which may display similar aggregate size in suspension, when studied by other techniques such as dynamic light scattering for example.

The calculation results for specific surface area of TiO$_2$ powders, expressed in m$^1$ and in m$^2$/g, together with uncertainty estimations, are gathered in the following table. The diameter calculated in the last column corresponds to the size of dense, perfectly monodisperse and spherical TiO$_2$ nanoparticles that would exhibit the same mean surface area.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Sample} & \text{Lim I(q)$^4$} & \Sigma & \text{Specific surface area} & \text{error on plateau} & \text{Equivalent diameter for spheres [nm]} \\
\hline
\text{NM103} & 15.9 & 2.16E+08 & 51.1 & +/-1.8 & +/-6.9 & 28 \\
\hline
\end{array}
\]

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

**Attached full study report**
- Remarks: Dispersion protocol

**Attached document**
- Remarks: Data in the report and details protocol in annex

**Applicant's summary and conclusion**

**Conclusions**
see the endpoint: comparison between SAXS and BET

**Cross-reference to other study**
Endpoint study record: Specific surface area by BET 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

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

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 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 needs 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

Identifier Reference Material/Nanomaterial
Identity NM-103

State of test material

other: fluffy powder

Confidential details on test material

Commercial name: UV TiTAN M262

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): 60

Results and discussions

The results on the specific surface area, pore volume and microporosity of the MN is summarized in Table 6.

Table 6: Summary of BET results on all three test materials and the internal reference.

<table>
<thead>
<tr>
<th>Material</th>
<th>BET surface</th>
<th>Total pore volume</th>
<th>Microsurface 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>NM103</td>
<td>50.835</td>
<td>0.2616</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

For TiO₂ nanomaterials (except for NM102) BET was straightforward and data treatment produced very good correlation coefficients. The nitrogen adsorption isotherms are plotted in Figure 4.10.
Figure 4.10: Isotherms of nitrogen sorption experiments at 77K for the TiO₂ nanomaterials. The sample numbers are mentioned in the title of each plot.

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: compraison 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
Data source

Reference

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

Data access

other: owner: NANOGENOTOX

Materials and methods


Remarks: Dispersion protocol


Remarks: Data in the report and details porocol 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</th>
<th>SAXS surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m²/g</td>
<td>m²/g</td>
</tr>
<tr>
<td>NM103</td>
<td>50.835</td>
<td>51.1(1.8)</td>
</tr>
</tbody>
</table>

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 twodifferent 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 Institute of Energy and Environmental Technology (IUTA)_

Administrative Data

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

**Study result type** experimental result

Data source

Reference

**Reference type** study report

**Author** Carmen Nickel, Bryan Hellack, Stefan Gartiser, Felicitas Flach, Andreas Schiwy, Hanna Maes, Andreas Schäffer, Stephan Gabsch, Michael Stintz, Lothar Erdi, Thomas A.J. Kuhlbusch

**Year** 2011

**Title** Fate and behaviour of TiO2 nanomaterials in the environment, influenced by their shape, size and surface area

**Testing laboratory** IUTA e.V. Duisburg, Hydrotex GmbH, Freiburg, RWTH Aachen, TU Dresden, Universitätsklinikum Heidelberg, Universität Duisburg-Essen, Germany

**Report no.** FKZ 3709 65 417

Materials and methods

Methods

other: DLS

**Used Protocols**
Standard operating procedure – Preparing Titanium dioxide suspensions in deionised water 1. Aim of the SOP 2. Background 3. Preliminary results 4. Preparing suspension 1. Aim of the Standard Operating Procedure (SOP) The aim of this Standard Operating Procedure is the preparation of a stable nanoscale Titanium dioxide suspension for environmental testing within the Project 3709 65 417 and afford reproducible results in different laboratories (comprehensible proceedings). The SOP describes the proceedings which are suitable for preparing a stable TiO2 nanoparticle suspension in this project for P25, PC105 and UV Titan M262. 2. Background Suspension Requirements - The suspension must be stable at least for 24 h (a variance of 10% is accepted). - An appropriate stability of a suspension is declared as a constant particle size distribution, concentration and zeta potential. Stability criteria - Optical observation (no visible sedimentation of the particles) - Size of the particles in the suspension - Zeta potential - Particle concentration - pH value of the suspension - Conductance of the suspension Necessary Instruments - A sensitive analytical balance. - Sonication equipment with sufficient rated power. - Sensitive instrument detecting the particle size distribution and the zeta potential in aqueous media. Used instruments In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanoparticle in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter / Zeta Sizer ZS - Malvern Instruments; Nanophox –
Sympatec, size only). 3. Preliminary results Preliminary results show that a sufficient stability is warranted if the suspension (100 mg TiO2 material / 100 mL deionised water in a 250 mL beaker glass) was sonicated for 10 minutes. 4. Preparing suspension - For preparing suspension deionised water was used (pH 5.0 - variance of 10%). - A defined amount of the nanomaterial - here 100 mg of the solid material was weighted in a 250 mL beaker glass (a variance of 1% is accepted). - After this 100 mL of deionised water was carefully added to the material. - The beaker glass with the nanoparticle suspension was sonicated with a Bandelin Sonoplus HD 2200 ultrasonic homogeniser for 10 minutes* with a pulse of 0.2 / 0.8. - The horn of the ultrasonic homogeniser was dipped into the suspension and placed in the middle of the beaker glass with a distance between horn and bottom of the beaker glass of approximately 1 cm. - For sonication the beaker glass with the suspension was put in a bigger beaker glass with cold/ice water to minimize the heating of the suspension during the sonication. - After use the horn was cleaned with ethanol and afterwards with deionised water. - After sonication the suspension was characterised to its size distribution – using a DLS instrument. Note: the sonication time must be adapted to the volume of the prepared suspension, diameter of the beaker glass, the concentration of the nanoparticles and the rated power of the ultrasonic instrument.

Data gathering

Instruments
In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanoparticle in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter / Zeta Sizer ZS - Malvern Instruments; Nanophox – Sympatec, size only).

Test materials

State of test material

dispersion

Results and discussions

Remarks on results including tables and figures
In suspension a Zeta-Potential of > +25 mV at pH 5, after 10 min sonication using an ultrasonic homogenizer (Bandelin SONOPlus HD2200, VST 70, pulse 02/08) was detected.
Overall remarks, attachments

Attached background material
diagramme_zetapotential.docx

Endpoint study record: Zeta potentia 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
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
Remarks on results including tables and figures
The zeta potential, measured in water, shows for NM-103 particles a negative zeta potential across all concentrations tested. In general, concentrated dispersions appeared to have more stable aggregates than diluted suspensions.

Table 1: Zeta Potential characterization by DLS

<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>10</td>
<td>-26.2</td>
<td>-24.4</td>
<td>-27.7</td>
<td>-7.97</td>
<td>-7.71</td>
</tr>
<tr>
<td>1</td>
<td>-25.0</td>
<td>-21.8</td>
<td>-23.2</td>
<td>-25.9</td>
<td>18.9</td>
</tr>
<tr>
<td>0.1</td>
<td>-19.6</td>
<td>-43.4</td>
<td>-19.7</td>
<td>-23.9</td>
<td>-10.0</td>
</tr>
<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 2: Conductivity characterization by DLS

<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>
</tr>
<tr>
<td>10</td>
<td>0.01640</td>
<td>0.01390</td>
<td>0.04480</td>
<td>0.01110</td>
<td>0.02430</td>
</tr>
<tr>
<td>1</td>
<td>0.01410</td>
<td>0.00606</td>
<td>0.01170</td>
<td>0.00621</td>
<td>0.02710</td>
</tr>
<tr>
<td>0.1</td>
<td>0.00406</td>
<td>0.00654</td>
<td>0.00989</td>
<td>0.01820</td>
<td>0.01340</td>
</tr>
<tr>
<td>0.01</td>
<td>0.01980</td>
<td>0.00698</td>
<td>--</td>
<td>0.01130</td>
<td>0.00548</td>
</tr>
</tbody>
</table>
Overall remarks, attachments
Attached background material table zeta potential.doc

**Endpoint study record: Zeta potential by Institute of Energy and Environmental Technology (IUTA) (2)**

**Administrative Data**

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

**Data source**

**Reference**

- **Reference type**: study report
- **Author**: Carmen Nickel, Bryan Hellack, André Nogowski, Frank Babick, Michael Stintz, Hanna Maes, Andreas Schäffer, Thomas Kuhlbusch
- **Year**: 2013
- **Title**: Mobility, fate and behaviour of TiO2 nanomaterials in different environmental media
- **Testing laboratory**: IUTA e.V. Duisburg, TU Dresden, RWTH Aachen, Universität Duisburg-Essen, Germany
- **Report no.**: FKZ 3710 65 414

**Materials and methods**

**Methods**

- DLS and micro-electrophoresis by using video microscopy.

**Used Protocols**

Standard operating procedure – Preparing Titanium dioxide suspensions in deionised water 1. Aim of the SOP 2. Background 3. Preliminary results 4. Preparing suspension 1. Aim of the Standard Operating Procedure (SOP) The aim of this Standard Operating Procedure is the preparation of a stable nanoscale Titanium dioxide suspension with minimal invasive methods to minimize surface degradation within the Project 3710 65 414 and afford reproducible results in different laboratories (comprehensible proceedings). The SOP describes the preparation steps for a stable TiO2 nanomaterial suspension in this project for UV Titan M262 2. Background Suspension Requirements - The suspension must be stable at least for 24 h (a variance of 10% is accepted) - An appropriate stability of a suspension is declared as a constant particle size distribution, concentration and zeta potential. Stability criteria - Optical observation (no visible sedimentation of the particles) - Size of the particles in the suspension - Zeta potential - Particle concentration - pH value of the suspension - Conductance of the suspension Necessary instrumentation - A sensitive analytical balance. - Sonication equipment with sufficient rated power. - Sensitive instrument detecting the particle size distribution and the zeta potential in aqueous media. Used instrumentation In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanomaterial in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter coupled with electrophoretic light scattering for the calculation of the zeta potential of the suspended particles) and micro-electrophoresis by using video microscopy. 3. Preliminary results Preliminary results show that a sufficient stability is warranted if the suspension (10 mg TiO2 material / 100 mL deionised water in a 250 mL beaker glass) was sonicated for 2 min for 100 mL or 1 min for 50 mL and stirred for 60 min. 4. Preparing suspension - For preparing suspension deionised water was used (pH 5.0 - variance of 10%). - A defined amount of the nanomaterial - here 10 mg of the solid...
material was weighted in a 250 mL beaker glass (a variance of 1% is accepted). - After this 100 mL of deionised water was carefully added to the material. - The beaker glass with the nanomaterial suspension was sonicated with a Bandelin Sonoplus HD 2200 ultrasonic homogeniser for 2 minutes* with a pulse of 0.2 / 0.8. - The horn of the ultrasonic homogeniser was dipped into the suspension and placed in the middle of the beaker glass with a distance between horn and bottom of the beaker glass of approximately 1 cm. - For sonication the beaker glass with the suspension was put in a bigger beaker glass with cold/ice water to minimize the heating of the suspension during the sonication. - Afterwards the suspension was stirred for 60 min using a magnetic stirrer. - After sonication the suspension was characterised to its size distribution and zeta potential. Note: the sonication time must be adapted to the volume of the prepared suspension, diameter of the beaker glass, the concentration of the nanomaterials and the rated power of the ultrasonic instrument.

Data gathering

Instruments

In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanomaterial in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter coupled with electrophoretic light scattering for the calculation of the zeta potential of the suspended particles) and micro-electrophoresis by using video microscopy.

Test materials

State of test material
dispersion

Any other information on materials and methods incl. tables

Suspension, particles were suspended in DI water with a pH of 5. The pH was stepwise adjusted using 0.1 M HCl and 0.1 M NaOH and the coating stability was analysed. As basic conditions DI water was used and the change of the zeta potential was detected as function of the pH and afterwards as function of ionic strength and dissolved organic carbon (DOC) content (0,01 M; 0,001 M; 0,0001 M CaCl2 solution and 2,5 mg/L, 5 mg/L and 10 mg/L DOC).

Results and discussions

Remarks on results including tables and figures

In suspension a Zeta-Potential of > +38 mV at pH 5, 1min sonication using an ultrasonic homogenizer (Bandelin SONOPlus HD2200, VST 70, pulse 02/08) and 60 min stirring was determined; n = 3. Zeta potential measurements as function of the pH were conducted- The pH was adjusted stepwise from acidic to basic conditions using 0.1 M HCl and 0.1 M NaOH. The iso electric point (IEP) was determined between pH 8-9 (Figure 1). A hysteresis was determined (Figure 2) when the pH was adjusted stepwise from acidic to basic and back to acidic conditions. The material lost his hydrophobic behaviour after it was wetted with water and after suspension preparation. By using the established SOP nearly all of the Dimethicon from the surface of the nanomaterials was released. On average 87 % were detected in the supernatant of the suspension by ICP MS. The IEP and ICP measurements indicate that the aluminium oxide coating was not significantly affected. The zeta potential measurements detected an IEP around pH 8 – 9 which is in accordance with the IEP of aluminium oxide (Shin et al. 2006; Kosmulski 2006) – (final report FKZ (UFOPlan) 3710 65 414). With increasing ionic strength the IEP was shifted to more basic conditions. At the highest test concentration of CaCl2 no IEP was detected. Agglomeration of the suspension was visually detected. With addition of DOC for all tested concentration the zeta potential was shifted to a negative value for all tested pH values. No IEP can be detected. The zeta potential and
agglomerate size in suspension was stable over the whole pH range. If both CaCl2 and DOC were added, the zeta potential was negative and the IEP was not detected.

Test Substance/Item

Figure 9: Zeta potential of UV Titan M262 as function of the pH in DI water, pH was adjusted using 0.1M HCl and 0.1M NaOH.

Figure 10: Zeta potential of UV Titan M262 as function of the pH in DI water, pH was adjusted using 0.1M HCl and 0.1M NaOH. First step, titration from acidic to basic conditions, second step titration from basic to acidic conditions, average measurement duration 3.5h.
Table 5: Zeta potential measurements of NM103 suspensions with varying pH (4.5 – 10) and CaCl\(_2\) concentration (0.0001M, 0.001M and 0.01M); IEP = isoelectric point; n = 3.

<table>
<thead>
<tr>
<th>pH</th>
<th>NM103</th>
<th>DI water</th>
<th>0.0001M CaCl(_2)</th>
<th>0.001M CaCl(_2)</th>
<th>0.01M CaCl(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zeta Potential (mV)</td>
<td>SD</td>
<td>Zeta Potential (mV)</td>
</tr>
<tr>
<td>4.5</td>
<td>37.7</td>
<td>1.8</td>
<td>34.6</td>
<td>3.3</td>
<td>37.3</td>
</tr>
<tr>
<td>5</td>
<td>32.1</td>
<td>1.8</td>
<td>34.8</td>
<td>2.7</td>
<td>39.5</td>
</tr>
<tr>
<td>6</td>
<td>25.3</td>
<td>1.6</td>
<td>30.8</td>
<td>2.6</td>
<td>37.3</td>
</tr>
<tr>
<td>7</td>
<td>12.3</td>
<td>8.4</td>
<td>26.4</td>
<td>1.2</td>
<td>31.4</td>
</tr>
<tr>
<td>8</td>
<td>-5.8</td>
<td>5.5</td>
<td>14.0</td>
<td>5.9</td>
<td>18.7</td>
</tr>
<tr>
<td>9</td>
<td>-30.8</td>
<td>0.8</td>
<td>-0.5</td>
<td>9.8</td>
<td>9.8</td>
</tr>
<tr>
<td>10</td>
<td>-24.4</td>
<td>2.1</td>
<td>-16.0</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>9</td>
<td>-11.9</td>
<td>3.8</td>
<td>-13.4</td>
<td>4.0</td>
<td>3.1</td>
</tr>
<tr>
<td>8</td>
<td>12.6</td>
<td>2.0</td>
<td>-7.1</td>
<td>6.6</td>
<td>5.3</td>
</tr>
<tr>
<td>7</td>
<td>23.9</td>
<td>4.7</td>
<td>10.7</td>
<td>4.9</td>
<td>15.7</td>
</tr>
<tr>
<td>6</td>
<td>35.1</td>
<td>0.9</td>
<td>24.6</td>
<td>1.8</td>
<td>28.0</td>
</tr>
<tr>
<td>5</td>
<td>37.0</td>
<td>0.0</td>
<td>32.1</td>
<td>0.4</td>
<td>36.4</td>
</tr>
<tr>
<td>4.5</td>
<td>0.0</td>
<td>0.0</td>
<td>33.8</td>
<td>0.5</td>
<td>37.6</td>
</tr>
<tr>
<td>IEP</td>
<td>8 ... 7</td>
<td>9.5 ... 8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 11: Zeta potential for NM103 and NM104 in CaCl\(_2\) solution, ELS (continuous titration from acid to basic); n = 3.
Figure 12: Zeta potential as function of pH for NM103 (left) and NM104 (right) dispersed according to the SOP in DI-water, measured with ELS (continuous titration: acid → basic → acid), 3 independent sample preparations.

Table 6: Zeta potential measurements of NM103 (UV Titan M262) suspensions with varying pH (4.5 – 10) and DOC content (2.5 mg/L, 5mg/L and 10 mg/L); IEP = isoelectric point; n = 3.

<table>
<thead>
<tr>
<th>NM103</th>
<th>DI water</th>
<th>2.5 mg/L NOM</th>
<th>5 mg/L NOM</th>
<th>10 mg/L NOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Zeta potential (mV)</td>
<td>SD</td>
<td>Zeta potential (mV)</td>
<td>SD</td>
</tr>
<tr>
<td>4.5</td>
<td>38.8</td>
<td>1.3</td>
<td>-13.3</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>37.7</td>
<td>1.8</td>
<td>-15.3</td>
<td>3.9</td>
</tr>
<tr>
<td>6</td>
<td>32.1</td>
<td>1.8</td>
<td>-20.9</td>
<td>1.6</td>
</tr>
<tr>
<td>7</td>
<td>25.3</td>
<td>1.6</td>
<td>-27.8</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>12.3</td>
<td>8.4</td>
<td>-29.0</td>
<td>2.7</td>
</tr>
<tr>
<td>9</td>
<td>-5.8</td>
<td>5.5</td>
<td>-29.3</td>
<td>3.4</td>
</tr>
<tr>
<td>10</td>
<td>-30.8</td>
<td>0.8</td>
<td>-27.2</td>
<td>6.4</td>
</tr>
<tr>
<td>9</td>
<td>-24.4</td>
<td>2.1</td>
<td>-28.4</td>
<td>4.6</td>
</tr>
<tr>
<td>8</td>
<td>-11.9</td>
<td>3.8</td>
<td>-30.3</td>
<td>2.8</td>
</tr>
<tr>
<td>7</td>
<td>12.6</td>
<td>2.0</td>
<td>-33.7</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>23.9</td>
<td>4.7</td>
<td>-30.3</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>35.1</td>
<td>0.9</td>
<td>-12.5</td>
<td>7.4</td>
</tr>
<tr>
<td>4.5</td>
<td>37.0</td>
<td>0.0</td>
<td>-6.4</td>
<td>1.7</td>
</tr>
<tr>
<td>IEP</td>
<td>9 ... 8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Overall remarks, attachments

Attached background material
diagramm zetapotential_UV Titan M262_371065414_18062012.doc

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 Author Karl Franzens, Dr. Eva Roblegg and Sandra Blass
Title Sponsorship Program: Titanium Dioxide Report

Data source

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 of the particle characterization of NM103 in different biological media

<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>Potential Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQ Wasser</td>
<td>973,2</td>
<td>671,6</td>
<td>0,287</td>
<td>40,4</td>
<td>5,27</td>
</tr>
<tr>
<td>PBS</td>
<td>1977</td>
<td>1397</td>
<td>0,255</td>
<td>-35,5*/9,04</td>
<td>-19,3</td>
</tr>
<tr>
<td>DMEM + L-Glutamine</td>
<td>2255</td>
<td>1665</td>
<td>0,256</td>
<td>-19,9*/11,1</td>
<td>-9,46</td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>1040*/4593</td>
<td>828,8</td>
<td>0,269</td>
<td>-8,8</td>
<td>9,41</td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>991,1</td>
<td>653,2</td>
<td>0,293</td>
<td>-10,8</td>
<td>12</td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>1156</td>
<td>683,3</td>
<td>0,369</td>
<td>-8,47</td>
<td>13,6</td>
</tr>
</tbody>
</table>

0,4 mg/ml TiO2 particles (NM103, 20 nm, hydrophobic rutile, Sachtleben M262)
untreated

<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>Potential Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min Sonifier (40% Amplitude)</td>
<td>0,4 mg/ml TiO2 particles (NM103, 20 nm, hydrophobic rutile, Sachtleben M262)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
0.4 mg/ml TiO2 particles (NM103, 20 nm, hydrophobic rutile, Sachtleben M262) 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>765,3/5041</td>
<td>596,9</td>
<td>0,393</td>
<td>39,1</td>
<td>6,08</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>1449/5037</td>
<td>1350</td>
<td>0,25</td>
<td>-20,9</td>
<td>11,2</td>
<td></td>
</tr>
<tr>
<td>DMEM + L-Glutamin</td>
<td>2916</td>
<td>2268</td>
<td>0,264</td>
<td>-8,76</td>
<td>15,4</td>
<td></td>
</tr>
<tr>
<td>DMEM + 1% FBS</td>
<td>684,1/4946</td>
<td>526,8</td>
<td>0,317</td>
<td>-10,0</td>
<td>15,4</td>
<td></td>
</tr>
<tr>
<td>DMEM + 5% FBS</td>
<td>1079</td>
<td>656,9</td>
<td>0,367</td>
<td>-13,7</td>
<td>20,3</td>
<td></td>
</tr>
<tr>
<td>DMEM + 10% FBS</td>
<td>1155/262,3</td>
<td>570,3</td>
<td>0,417</td>
<td>-11,8</td>
<td>14,1</td>
<td></td>
</tr>
</tbody>
</table>

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>
</tr>
</thead>
<tbody>
<tr>
<td>MQ Wasser</td>
<td>17297</td>
<td>621053</td>
<td>1503706</td>
<td>0 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>446</td>
<td>2003</td>
<td>6811</td>
<td>1 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>530</td>
<td>3355</td>
<td>7986</td>
<td>2 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>689</td>
<td>4255</td>
<td>9195</td>
<td>3 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>1134</td>
<td>4681</td>
<td>9988</td>
<td>4 min US</td>
</tr>
<tr>
<td>MQ Wasser</td>
<td>1808</td>
<td>5083</td>
<td>10590</td>
<td>5 min US</td>
</tr>
</tbody>
</table>

Overall remarks, attachments

Attached background material
Uni Graz_Robleeg_Agglomeration NM103.docx
**Endpoint study record: Zeta potential_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**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author</strong></td>
<td>Keld Alstrup Jensen</td>
</tr>
<tr>
<td><strong>Title</strong></td>
<td>Deliverable 4.5: Nanomaterial datasets with requested physicochemical properties. Surface charge, hydrodynamic size and size distributions of NM in aqueous suspensions by zetametry, dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS)</td>
</tr>
<tr>
<td><strong>Bibliographic source</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Testing laboratory</strong></td>
<td>CEA (F)</td>
</tr>
<tr>
<td><strong>Owner company</strong></td>
<td>NANOGENOTOX</td>
</tr>
<tr>
<td><strong>Company study no.</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Data access**

other: data owner: NANOGENOTOX

**Data protection claimed**

yes, but willing to share

**Materials and methods**

**Methods**

Laser-Doppler-Electrophoresis

**Details on methods and data evaluation**

- Electrophoretic mobility is measured by a combination of laser Doppler velocimetry, a technique based on the phase shift of the laser beam induced by the movement of particles under an electric field, and phase analysis light scattering (patented M3-PALS technique).
- In this “mixed mode measurement” (M3), the measurement consists of the application of an alternative electric field in two modes, a fast field reversal mode, and a slow field reversal mode. The light scattered at an angle 17° is combined with the reference beam and the resulting signal is treated by the computer.
- During the fast field reversal mode, the electro-osmose effect is negligible, allowing to determine an accurate mean zeta potential, whereas the slow field reversal mode helps modelling the distribution of potentials.
- More details on the results of zeta potential measurements with the M3-PALS technique are available in the documentation from Malvern Instruments and can be downloaded from http://www.malvern.com, application library section.

**Used Protocols**

The SOP is developed by CEA and it is different from the Nanogenotox SOP. The details of the procedure can be found in the attached files with SOP1) Sample preparation:Samples for zeta potential measurements are prepared as aqueous suspensions of 0.5 g/L for TiO2 nanomaterials with constant ionic strength of 0.036 mol/L (monovalent salt) and controlled pH. They are prepared by dilution of concentrated sonicated stock suspensions of 10 g/L into pH and ionic strength controlled “buffers”
prepared by addition of HNO3, NaOH and NaNO3 in various proportions. 20 mL of stock suspensions of 10 g/L NM in pure water are prepared as follows: o 200 mg of NM are weighed and introduced in a 20 mL gauged vial (with protective gloves, mask and glasses, and damp paper towel around the weigh-scale). o The 20 mL gauged vial is completed with ultrapure water (MilliQ). o The suspension is transferred into a flask suitable for sonication (a 40 mL large-neck glass flask of internal diameter 38 mm was used, height of 20 mL liquid 20 mm), making sure that all the settling material is recovered. o The suspension is dispersed by ultrasonication for 20 min at 40% amplitude in an ice-water bath. Probe, sample and bath are placed in a sound abating enclosure, and inside a fume hood. 2) Preparation of “buffer” solution: Denominated “buffer” solutions are aqueous ionic solutions of Na+, H+, NO3- and OH-, designed to display the same ionic strength with a modulated pH. o A first set of concentrated buffer solutions (0.1 mol/L of salt, various pH) are prepared by addition of HNO3, NaOH and NaNO3 in various proportions in ultrapure water. o Then 20 mL of these concentrated buffers are poured into 50 mL gauged vials completed with ultrapure water, giving a new set of buffers with a salt concentration of 0.04 mol/L and a pH ranging from 1.5 to 12.5. The combination of the two buffers gives access to the necessary intermediate pH. o By this procedure, acidic buffers contain 0.04 mol/L of NO3- and various ratios of Na+/H+ as counter ions; likewise, basic buffers contain 0.04 mol/L of Na+ and various ratios of NO3-/OH-. 3) Preparations of suspensions for zeta potential measurements and determination of isoelectric point: In this SOP Zeta potential measurements are performed on 0.5 g/L suspensions for TiO2 samples. o For TiO2 samples, freshly sonicated stock suspensions are first two-fold diluted in ultrapure water to get 5 g/L concentrated suspensions. o Series of samples are prepared by addition of 400 µL of concentrated NM suspension and 3.6 mL of 0.04 mol/L buffer solutions in a 5 mL glass flask. o This leads to samples of 0.5 g/L TiO2 and a constant ionic concentration of 0.036 mol/L in monovalent salt. o For each NM, an additional sample is prepared in MilliQ or Nanopure water with the same NM concentrations, i.e. by addition of 400 µL of concentrated NM suspension and 3.6 mL of water.

**Used Protocols: attached files**

<table>
<thead>
<tr>
<th>Attached document</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2_WP4_sOPs report: ENV/JM/MONO(2015)17/ANN1</td>
<td>Dispersion protocol</td>
</tr>
<tr>
<td>D4.5_ZETA_DLS_SAXS_analysis: ENV/JM/MONO(2015)17/ANN6</td>
<td>Data in the report and details porocol in annex</td>
</tr>
</tbody>
</table>

**Data gathering**

**Instruments**

- Zetasizer Nano ZS (e.g. Malvern Instruments), equipped with laser 633 nm
- Autotitrator (Malvern MPT-2) –optional for automatic determination of IEP
- Malvern software (DTS 5.03 or higher) installed on a computer to control the Zetasizer
- Clear, disposable zeta cells (DTS1061 - DTS1060C)

**Calibration**

o Equilibrium pH of the suspensions are measured and considered as pH values for the reported results. o The suspension to be characterized by zetametry are inserted in Malvern patented folded capillary cells with gold electrodes (volume 0.75 to 1 mL), DTS1061. o Zeta measurements (electrophoretic mobility) are performed on the “general purpose” mode at 25°C with automatic optimization of laser power, voltage settings, the number of runs (10 - 100) and run duration, and repeated 3 times with no equilibration time as the sample is already at ambient temperature. o The Smoluchowski model (F(κa)=1.5) was used, considering the high polarity of aqueous solvent, and hence a thin double layer around the particles. o For the dispersant, the refractive index R, absorption Rabs, viscosity and dielectric properties considered are the ones of pure water. o The parameters used for dispersant and material properties are available in the attached file with the SOP for Zetametry. o For each suspension of known pH, fixed ionic strength and fixed NM concentration, the measurements for determining the zeta potential are performed on a general
purpose mode with automatic determination of measurement parameters. 3 Three measurements are performed and averaged for reporting. 3 For unstable samples, measurements are performed on supernatants. 3 Zeta potentials are then plotted against pH to determine the stability domains and isoelectric points (IEP)-see attached file with the figure.

Reproducibility
3 Three measurements are performed and averaged for reporting. 3 For unstable samples, measurements are performed on supernatants. 3 The reported value is the average of zeta potential values from the 3 measurements (determined during the fast field reversal step), with possible exclusion of diverging data.

Test materials

Test material equivalent to submission substance identity
3 yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM-103

State of test material
dispersion

Confidential details on test material
Commercial name: UV Titan M262

Results and discussions

Zeta potential
Results of zeta potential vs. pH are gathered for all TiO₂ NM in Figure 0.13. The corresponding IEP appear in the insert table. Half-filled symbols represent unstable samples which are strongly aggregated and sediment. In that case, zeta potential is measured on supernatants.

Figure 0.13: Zeta potential as a function of pH for TiO₂ NP suspensions (0.5 g/L) in constant ionic strength aqueous media (0.036 mol/L HNO₃/NaOH), highlighting domains of stability for acidic pH and instability around isoelectric points (values in insert). Measurements on supernatant for fast sedimenting suspensions appear as half-filled dots.
The tested TiO$_2$ NM (NM102, NM103, NM104, and NM105) form stable suspensions at acidic pH (below pH 4) where all NM have high positive charge, exceeding 30 mV. Negative zeta potentials, lower than -30 mV, were observed at high pH values (from 2 pH units above the IEP). The IEP obtained for NM102 and NM105 (pH 6 to 7), are in accordance with expected values for TiO$_2$ nanomaterials. The higher IEP of pH 8.2 observed for NM103 and NM104 can be explained by the presence of an Al$_2$O$_3$ coating on the surface of these nanoparticles. In addition, NM103 and NM104 were unstable at pH levels around 6 despite measuring a zeta-potential of app. +40 mV on their supernatant. This may be due to surface heterogeneities of these NM.

The average aggregate sizes measured by DLS increase when increasing pH from the acidic stability domain toward the isoelectric points. This is consistent with theory where agglomeration and hence average size will increase with decreasing surface charge. For higher pH, suspensions are not stable and sediment rapidly. Stability should, however, be regained at high pH values, where the negative zeta potentials became smaller than -40 mV.

In medium (specify) suspensions (0.5 g/L) in constant ionic strength aqueous media (0.036 mol/L HNO3/NaOH)

Remarks on results including tables and figures

Overall remarks, attachments

Overall remarks

NM 102 form stable suspensions at acidic pH (below pH 4) where all NM have high positive charge, exceeding 30 mV. Negative zeta potentials, lower than -30 mV, were observed at high pH values (from 2 pH units above the IEP). The IEP obtained for NM102 (pH 6 to 7), are in accordance with expected values for TiO$_2$ nanomaterials.

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

Executive summary

For each suspension of known pH, fixed ionic strength and fixed NM concentration, the measurements for determining the zeta potential are performed on a general purpose mode with automatic determination of measurement parameters. Three measurements are performed and averaged for reporting. For unstable samples, measurements are performed on supernatants. Zeta potentials are then plotted against pH to determine the stability domains and isoelectric points (IEP). NM 102 form stable suspensions at acidic pH (below pH 4) where all NM have high positive charge, exceeding 30 mV. Negative zeta potentials, lower than -30 mV, were observed at high pH values (from 2 pH units above the IEP). The IEP obtained for NM102 (pH 6 to 7), are in accordance with expected values for TiO$_2$ nanomaterials.

Cross-reference to other study
4.30 Surface chemistry

Endpoint study record: RB adsorption constant by University of Graz

Administrative Data

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

Study result type experimental result

Data Author Karl Franzens, 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

other: Rose Bengal adsorption method

Overall remarks, attachments

Overall remarks

NM 103 demonstrated a lower hydrophilic surface with a binding constant of K=0.092 ± SD 0.020

Endpoint study record: Surface chemistry by Institute of Energy and Environmental Technology (IUTA)

Administrative Data

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

Study result type experimental result

Data source

Reference

Reference type study report

Author Carmen Nickel, Bryan Hellack, André Nogowski, Frank Babick, Michael Stintz, Hanna Maes, Andreas Schäffer, Thomas A.J. Kuhlbusch

Year 2013

Title Mobility, fate and behaviour of TiO2 nanomaterials in different environmental media

Testing laboratory IUTA e.V., Duisburg, TU Dresden, RWTH Aachen, Center for Nanointegration Duisburg-Essen (CENIDE), Universität Duisburg-Essen, Germany

Report no. FKZ 3710 65 414

Materials and methods
Methods
other: SEM EDX, ICP-OES

Results and discussions
Functional groups (for each group: mean, standard dev)
NM103 materials are covered by hydrophobic layer of dimethicone (C2H6OSi)n.

Figure 10: SEM / EDX scan of the dry powder of NM103.
Overall remarks, attachments
Attached background material
IUTA SEM EDX NM103.docx

4.31 Dustiness

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

Administrative Data

<table>
<thead>
<tr>
<th>Purpose flag</th>
<th>key study (X) robust study summary ( ) used for classification ( ) used for MSDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study result type</td>
<td>experimental result</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
</tr>
<tr>
<td>Reference type</td>
</tr>
</tbody>
</table>
Materials and methods

Methods

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

o 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). o 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. o 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. o The drum was electrically grounded as prescribed by EN 15051. o 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). o The inlet air to the drum was controlled at 50 % RH and HEPA-filtered to ensure no particle background. o 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. o 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. o The saturation test was performed using 2 grams of powder and rotation for 60 seconds. o Then the actual triplicate tests were completed using 6 grams of test material per run. o After each run the drum was emptied by pouring out the residual powder and gently tapping the drum three times with a rubber hammer. o 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. o 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-103

**State of test material**
other: fluffy powder

**Confidential details on test material**
Commercial name: UV Titan M262

Results and discussions

*Table 1*: Number of dust particles and mass-based dustiness indexes of TiO$_2$ 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>Dustiness index</th>
<th>Mass (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number (1/mg)</td>
<td>Respirable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPC</td>
<td>Inspirable</td>
</tr>
<tr>
<td>NM-103</td>
<td>6</td>
<td>1.80E+07</td>
<td>9185 (±234)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>323 (±166)</td>
<td></td>
</tr>
</tbody>
</table>

Remarks on results including tables and figures

Figure 14 shows the particle number size distributions of aerosols generated during rotating drum dustiness testing of the TiO$_2$ samples. It is evident that all TiO$_2$ 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 15 and Figure 16 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 14: 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).

Figure 15: Dustiness ranking of inhalable dust for TiO2 nanomaterials (NM10x) and SAS (NM20x) nanomaterials as obtained with the small rotating drum method at NRCWE.
Figure 16: 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 details protocol 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>KA Jensen</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 4.6: Dustiness of NANOGENOTOX nanomaterials using the NRCWE small rotating drum and the INRS Vortex shaker</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>INRS (F)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D4.6</td>
</tr>
<tr>
<td>Owner company</td>
<td>Company study no.</td>
</tr>
</tbody>
</table>

Data access
other: 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 test powder 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 vial altogether with 5 g bronze beads (100 μm), used to agitate and de-agglomerate 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 versions were developed. The first version is devoted for real-time measurement using ELPI™ 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 ELPI™ 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 10^7 particles/cm^3 with high accuracy. ELPI™ (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 ELPI™ 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 test, 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 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 kilogram:

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 PVEGELMAN 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 NFT 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 setup (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

96
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 concentrationssubstrates used PVC GELMAN GLA-5000 5μm / 25 mmDifferent 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**

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Reference Material/Nanomaterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>NM-103</td>
</tr>
</tbody>
</table>

**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>
</tr>
</tbody>
</table>
Confidential details on test material
Commercial name: UV Titan M262

Results and discussions
Experimental data obtained with the Vs method are summarized in the Error! Reference source not found. below. 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>
<thead>
<tr>
<th>Sample (mg)</th>
<th>CPC (S.D)</th>
<th>T = 180 s</th>
<th>ELPI (S.D)</th>
<th>T = 3600 s CPC (S.D)</th>
<th>Respirable (S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM-103</td>
<td>216.8</td>
<td>5.4E+05</td>
<td>2.0E+06</td>
<td>1.9E+06</td>
<td>1.9E+04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0E+04</td>
<td>2.7E+05</td>
<td>1.7E+05</td>
<td>1.70E-02</td>
</tr>
</tbody>
</table>

* 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/cm³ for NM100, 101, 102 and 4.26 g/cm³ for NM103, 104, 105 based on Teleki et al. (2008); 2.2 g/cm³ for all NM20x; 1.75 g/cm³ for all NM40x based on Kim et al. (2009).

<table>
<thead>
<tr>
<th>Remarks on results including tables and figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>The following table provides information on the gravimetric water content (expressed in terms of the mass of water per unit mass of the dried sample in percentage) and bulk density of the nanomaterials in powders. The results were obtained in tests conducted in the INRS laboratory. Only TiO₂ and SiO₂ samples were treated. The water content was not determined for NTC samples as the conditions for the manipulation were not satisfactory, especially in terms of prevention of operator exposure.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample mass (mg)</th>
<th>Water content (wt % dry)</th>
<th>Bulk density (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM-103</td>
<td>126</td>
<td>2%</td>
<td>0.44</td>
</tr>
</tbody>
</table>

The Figure 17 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. The highest values were obtained with the SAS NM 203 (5.1E+04 mg/kg) while the lowest with the NTC NM400 (< 4.2E+02 mg/kg). 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 17: 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 18 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 NM 400 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.

In the following Figure 19 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 18: Number (1/mg) and respirable mass (mg/kg) dustiness indices of MN tested with the VS method.

Figure 19: Number (1/mg) dustiness indices for all MN tested with the VS method as measured by CPC. Comparison between two test times T for calculation: 180 and 3600 s.

Comparison between SD and VS method

Figure 20 and Figure 21 compares the respirable mass dustiness indices obtained according to the two methods (SD and VS) used in the project. Figure 21 is identical to Figure 20 except that the data are presented here according to the respirable mass indices ranked from the highest to the lowest for the method VS. It can be seen that the two methods do not lead to the same classification according to this indices. There are several reasons to explain this but this beyond the scope and objective of this report. Regarding the fact that the VS method lead to higher respirable mass indices than the SD method, it is probably because the VS method is a method that transmit more energy to the MN powder sample than
the SD method. However, it is not yet possible to quantify the energy input for both methods. Such issue needs to be investigated before standardized tests can be defined.

Figure 20: Comparison between respirable mass dustiness indices obtained with the small rotating drum (SD) and vortex shaker (VS) method. Errors bars on the SD values correspond to the reproducibility over 3 repeats. The NM40x samples have been tested with the VS method only.

As written in the introduction, 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.
Figure 21: Comparison between respirable mass dustiness indices obtained with the small rotating drum (SD) and vortex shaker (VS) method. MN samples are classified according to their highest respirable mass index to the lowest obtained with the VS method.

**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 porocol in annex

**Applicant's summary and conclusion**

**Conclusions**

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 to 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: Porosity by BET 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>study report</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>Testing laboratory</td>
<td>IMC-BAS (BG)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D4.4</td>
</tr>
<tr>
<td>Owner company</td>
<td></td>
</tr>
<tr>
<td>Company study no.</td>
<td>Report date</td>
</tr>
</tbody>
</table>

 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 (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 in layers 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 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

Identifier Reference Material/Nanomaterial

Identity NM-103

State of test material

other: fluffy powder
Confidential details on test material
Commercial name: UV TiTAN M262

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): 60

Results and discussions
The results on the specific surface area, pore volume and microporosity of the MN is summarized in Table 6.

Table 6: Summary of BET results on all three test materials and the internal reference.

<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>NM103</td>
<td>50.835</td>
<td>0.2616</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

For TiO₂ nanomaterials (except for NM102) BET was straightforward and data treatment produced very good correlation coefficients. The nitrogen adsorption isotherms are plotted in Figure 4.10.

![Figure 4.10: Isotherms of nitrogen sorption experiments at 77K for the TiO₂ nanomaterials. The sample numbers are mentioned in the title of each plot.](image)

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 porocel 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

Endpoint study record: Photocatalytic activity by Institute of Energy and Environmental Technology (IUTA)

Administrative Data

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

Data source

Reference
Author Carmen Nickel, Bryan Hellack, André Nogowski, Frank Babick, Michael Stintz, Hanna Maes, Andreas Schäffer, Thomas A.J. Kuhlbusch
Year 2013
Title Mobility, fate and behaviour of TiO2 nanomaterials in different environmental media
Testing laboratory IUTA e.V., TU Dresden, RWTH Aachen, Center for Nanointegration Duisburg-Essen, Universität Duisburg-Essen,
Report no. FKZ 3710 65 414

Materials and methods

Principles of method if other than guideline
H2O2 with and without UV irradiation was measured. For the measurement 30 μL of the particle suspension (final conc. 5 mg/L) is mixed with 30 μL DMPO (final conc. 0.05 M), analysed by EPR after irradiation with UV-light (UV Energy saving lamp Omnilux 25 Watt E27 3U 230V/50Hz AC, 6000 UV K, 22000 lm) for 10 min and compared to not irradiated samples.

Data gathering
Test materials
State of test material
dispersion
Figure 22: Hydroxyl radical generation of the coated titanium dioxide material NM104, NM103 and the uncoated P25 with and without UV irradiation, suspension preparation based on SOP, pH 5; mean values of $n = 3$.

Figure 46: EPR measurements of 100 mg/L P25 as positive control. Suspension preparation based on established SOP and 1 min vortexing, with 5 min UV irradiation; $n = 3$. 

Magnetic field in Gauss

- P25 UV-irradiation
- P25 no UV-irradiation
- NM-104 UV-irradiation
- NM-104 no UV-irradiation
- NM-103 UV-irradiation
- NM-103 no UV-irradiation

Intensity in a.u.

331.5 333.9 336.3 338.6
Overall remarks, attachments

Overall remarks
No hydroxyl radical generation was detected for the coated titanium dioxide materials with or without UV irradiation, independent on the type of suspension preparation (mixing or SOP).

Attached background material
IUTA Photoactivity.docx
4.35 Radical formation potential

*Endpoint study record: Radical formation potential by Institute of Energy and Environmental Technology (IUTA)*

**Administrative Data**

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

*Study result type* experimental result

**Data source**

*Reference*

*Reference type* study report

*Author* Carmen Nickel, Bryan Hellack, André Nogowski, Frank Babick, Michael Stintz, Hanna Maes, Andreas Schäffer, Thomas A.J. Kuhlbusch

*Year* 2013

*Title* Mobility, fate and behaviour of TiO2 nanomaterials in different environmental media

*Testing laboratory* IUTA e.V., Duisburg, TU Dresden, RWTH Aachen, 4 Center for Nanointegration Duisburg-Essen (CENIDE), Universität Duisburg-Essen, Germany

*Report no.* FKZ 3710 65 414

**Materials and methods**

*Details on methods and data evaluation*

In a first approach the hydroxyl radical generation (OH •) potential was detected (Table 3). Briefly, 50 μL of the particle suspension is mixed with 100 μL DMPO (0.05 M) and 50 μL of H2O2 (0.5 M), incubated in a dark, shaking water bath for 15 min at 37 °C. The presence of hydrogen peroxide (H2O2) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) in this method is especially sensitive for the detection of hydroxyl radicals (OH •) generated via Fenton-type reaction according to Shi et al. (2003). The signal intensity expressed as arbitrary unit (AU) of the DMPO-OH adduct was measured by EPR. As blank value dH2O was measured. Based on the mean of the blank (n = 3) a limit of detection (LOD) is calculated (MV blank + 3*standard deviation (SD) of the blank) and used for the data evaluation. The second analysis method, using CPH as the spin probe, showed a “surface reactivity” for the same materials. 50 μL of particle suspensions was mixed with 50 μL of the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrroldine hydrochloride (CPH) (1 mM), mixed with the chelator desferroxamin (0.1 mM) and incubated for 10 min at 37 °C. The ability to split H+ from the CPH molecule was measured. Due to the, with time increasing, blank EPR signal (dH2O) caused by an auto-oxidation of the spin probe (CPH) the results are expressed as blank corrected. The correction was done using the linear increase (R2 > 0.95) of the blank signal with time. Values higher than the time dependent blank values in respect to the standard deviation are named as reactive. The suspension was prepared using the established SOP and an alternative preparation with 1 min mixing without sonication to get information about the energy effect on the surface functionality. To get information about the influence of the energy input on the surface reactivity an alternative suspension preparation with only 1 min mixing using a vortex mixer was conducted.
Table 7: Hydroxyl radical generation (OH∙) potential in arbitrary unit (AU) of the coated titanium dioxide nanomaterials NM103 and NM104 and the uncoated P25 as photocatalytic material, suspension preparation based on SOP, pH 5; ± standard deviation of n = 3.

<table>
<thead>
<tr>
<th>DMPO</th>
<th>NM103</th>
<th>NM104</th>
<th>P25</th>
<th>Blank (dH₂O)</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean in AU (not blank corrected)</td>
<td>1833 ± 632</td>
<td>1490 ± 23</td>
<td>2407 ± 579</td>
<td>2298 ± 54</td>
<td>2460</td>
</tr>
<tr>
<td>OH∙ generation</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 8: Surface reactivity in arbitrary unit (AU) of the coated titanium dioxide nanomaterials NM103 and NM104 and the uncoated P25 as photocatalytic material, suspension preparation based on SOP; n = 3.

<table>
<thead>
<tr>
<th>CPH</th>
<th>NM103</th>
<th>NM104</th>
<th>P25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean in AU (blank corrected)</td>
<td>510</td>
<td>3322</td>
<td>1624</td>
</tr>
<tr>
<td>σ</td>
<td>580</td>
<td>1699</td>
<td>1446</td>
</tr>
<tr>
<td>Reactivity</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 9: Surface reactivity in arbitrary unit (AU) of the coated Titan materials NM103 and NM104, suspension preparation with 1 min mixing, n = 3.

<table>
<thead>
<tr>
<th>CPH</th>
<th>NM103</th>
<th>NM104</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean in AU (blank corrected)</td>
<td>330</td>
<td>-1049</td>
</tr>
<tr>
<td>σ</td>
<td>452</td>
<td>378</td>
</tr>
<tr>
<td>Reactivity</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Overall remarks, attachments

Overall remarks
1st approach: For none of the tested materials a hydroxyl radical generation was detected without UV irradiation. 2nd approach: NM103 showed no hydroxyl radical generation and no surface reactivity independent on the type of suspension preparation.

Attached background material
IUTA_radical formation.docx
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: Preliminary: Dispersion stability in by Institute of Energy and Environmental Technology (IUTA)*

**Administrative Data**

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

**Conclusions**

see agglomeration/aggregation and zeta potential by IUTA

*Endpoint study record: Dispersion stability in water by University of Vienna, Department of Environmental Geosciences*

**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.
- **Year**: (2012)
- **Title**: Testing the OECD selected alternative nano-TiO2 materials for dispersion stability, environmental behaviour and fate.
- **Type**: Project report
- **Owner**: University of Vienna, Department of Environmental Geosciences

**Conclusions**

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

Attached document 2: Project report TiO2 OECD BMVIT.pdf:

**Endpoint study record: Stabilisation in Complex Media by NANOGENOTOX**

**Administrative Data**

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

**Conclusions**

Preparing TiO2 nanoparticle (NP) suspensions displaying well-defined and reproducible dispersion state is a key feature to perform relevant toxicity experiments for environmental, animal, or human concerns. Relying on the evolution of surface charge with pH, and interactions between nanoparticles in their medium, we developed an optimized dispersion protocol involving a pH adjustment before addition of bovine serum albumin (BSA). It yielded highly dispersed and stable concentrated stock suspensions of TiO2 NP at pH 7. It was designed for four kinds of manufactured TiO2 nanomaterials and can be extended to a wide range of TiO2 NP. The suspensions studied here were characterized by small-angle X-ray scattering (SAXS), using a model quantitatively describing fractal aggregates. Results were correlated with dynamic light scattering (DLS) measurements. Moreover, the stability in a typical biological medium was assessed by diluting stock suspensions in Luria–Bertani (LB) medium. It resulted in highly dispersed and stable working suspensions. No sedimentation, followed by in situ DLS, was observed over 17 h for both the concentrated stock suspensions prepared according to the pH adjusted-BSA protocol and their dilution into LB medium.

Attached document: Guiot_ES&T 2012 (stability complex media Nanogenotox).pdf:
ENV/JM/MONO(2015)17/PART1/ANN23

**Attached document** D2_WP4 SOPs report: ENV/JM/MONO(2015)17/ANN1
5.1.6 Preliminary: Abiotic degradability and fate

*Endpoint study record: stability of coating by IUTA*

**Administrative Data**

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

Study result type: experimental result

**Conclusions**

The material lost his hydrophobic behaviour after it was wetted with water and after suspension preparation. By using the established SOP nearly all of the Dimethicon from the surface of the nanomaterials was released. On average 87% were detected in the supernatant of the suspension by ICP MS. The IEP and ICP measurements indicate that the aluminium oxide coating was not significantly affected. The zeta potential measurements detected an IEP around pH 8 – 9 which is in accordance with the IEP of aluminium oxide (Shin et al. 2006; Kosmulski 2006) – (final report FKZ (UFOPlan) 3710 65 414). With increasing ionic strength the IEP was shifted to more basic conditions. At the highest test concentration of CaCl2 no IEP was detected. Agglomeration of the suspension was visually detected. With addition of DOC for all tested concentration the zeta potential was shifted to a negative value for all tested pH values. No IEP can be detected. The zeta potential and agglomerate size in suspension was stable over the whole pH range. If both CaCl2 and DOC were added, the zeta potential was negative and the IEP was not detected. Associated background documents: Carmen Nickel, Bryan Hellack, Hanna Maes, Andreas Schäffer, Andre Nogowski, Frank, Babick, Michael Stintz, Thomas Kuhlbusch: Final report "Mobility, fate and behaviour of TiO2 nanomaterials in different environmental media " FKZ (UFOPlan) 3710 65 414), FEDERAL ENVIRONMENTAL PROTECTION AGENCY 2013

**Attached document 3: SOP preparing stock suspension coating experiments.pdf:**


5.2 Biodegradation

5.2.1 Biodegradation in water: screening tests

5.2.2 Biodegradation in water and sediment: simulation tests

5.2.3 Biodegradation in soil

5.2.4 Mode of degradation in actual use
5.3 Bioaccumulation

5.4 Transport and distribution

5.4.1 Adsorption / desorption

Endpoint study record: OECD106 Dispersion stability in by Institute of Energy and Environmental Technology (IUTA)

Administrative Data

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

Data source

Reference
Reference type study report
Author Carmen Nickel, Bryan Hellack, Stefan Gartiser, Felicitas Flach, Andreas Schiwy, Hanna Maes, Andreas Schäffer, Stephan Gabsch, Michael Stintz, Lothar Erdinger, Thomas A.J. Kuhlbusch
Year 2011
Title Fate and behaviour of TiO2 nanomaterials in the environment, influenced by their shape, size and surface area
Testing laboratory IUTA e.V. Duisburg, Hydrotex GmbH, Freiburg, RWTH Aachen, TU Dresden, Universitätsklinikum Heidelberg, Universität Duisburg-Essen, Germany
Report no. FKZ 3709 65 417

Materials and methods

Study type
adsorption/desorption

Media
soil

Type of method
batch equilibrium method

Test guideline
Qualifier according to
Guideline OECD Guideline 106 (Adsorption - Desorption Using a Batch Equilibrium Method)

Deviations

Study design

Test temperature
No specified conditions, the experiments were conducted under room conditions(15 – 25 °C).
Batch equilibrium or other method

Details on sampling

A Stock suspension was prepared using following SOP. Standard operating procedure – Preparing Titanium dioxide suspensions in deionised water 1. Aim of the SOP 2. Background 3. Preliminary results 4. Preparing suspension 1. Aim of the Standard Operating Procedure (SOP) The aim of this Standard Operating Procedure is the preparation of a stable nanoscale Titanium dioxide suspension for environmental testing within the Project 3709 65 417 and afford reproducible results in different laboratories (comprehensible proceedings). The SOP describes the proceedings which are suitable for preparing a stable TiO2 nanoparticle suspension. 2. Background Suspension Requirements - The suspension must be stable at least for 24 h (a variance of 10% is accepted). - An appropriate stability of a suspension is declared as a constant particle size distribution, concentration and zeta potential. Stability criteria - Optical observation (no visible sedimentation of the particles) - Size of the particles in the suspension - Zeta potential - Particle concentration - pH value of the suspension - Conductance of the suspension

Necessary Instruments - A sensitive analytical balance. - Sonoication equipment with sufficient rated power. - Sensitive instrument detecting the particle size distribution and the zeta potential in aqueous media. Used instruments In this Project sonication equipment (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) was used to disperse TiO2 nanoparticle in an aqueous suspension. The particle size and the zeta potential of the suspension were measured using DLS instruments (Delsa-Nano CS – Beckman Coulter / Zeta Sizer ZS - Malvern Instruments; Nanophox – Sympatec, size only).

3. Preliminary results Preliminary results show that a sufficient stability is warranted if the suspension (100 mg TiO2 material / 100 mL deionised water in a 250 mL beaker glass) was sonicated for 10 minutes. 4. Preparing suspension - For preparing suspension deionised water was used (pH 5.0 - variance of 10%). - A defined amount of the nanomaterial - here 100 mg of the solid material was weighted in a 250 mL beaker glass (a variance of 1% is accepted). - After this 100 mL of deionised water was carefully added to the material. - The beaker glass with the nanoparticle suspension was sonicated with a Bandelin Sonoplus HD 2200 ultrasonic homogeniser for 10 minutes* with a pulse of 0.2 / 0.8. - The horn of the ultrasonic homogeniser was dipped into the suspension and placed in the middle of the beaker glass with a distance between horn and bottom of the beaker glass of approximately 1 cm. - For sonication the beaker glass with the suspension was put in a bigger beaker glass with cold/ice water to minimize the heating of the suspension during the sonication. - After use the horn was cleaned with ethanol and afterwards with deionised water. - After sonication the suspension was characterised to its size distribution – using a DLS instrument. Note: the sonication time must be adapted to the volume of the prepared suspension, diameter of the beaker glass, the concentration of the nanoparticles and the rated power of the ultrasonic instrument.

Details on analytical methods

The soil / suspension mixture was centrifuged and the supernatant was analysed of its Titanium content, using ICP/OES after HCl, HNO3, HF digestion. Digestion Method Titantium dioxide in aqueous matrices is digested using a mixture of aqua regia and HF. Up to 10 mL of sample (depending on the type of sample) was pipetted into a PET test tube for centrifugation. Samples were concentrated to about 100 µL using a commercial concentration apparatus (Turbova, Zymark, Germany) at 70°C under a light nitrogen stream. After addition of aqua regia 2.4 mL HCl (36 - 38%, Ti < 0.2 ppb, J.T. Baker) and 0.8 mL HNO3 (69%, Ti < 0.5 ppb) 0.8 mL of HF (48%, Roth supra, Ti < 1 ppb) was added and the mixture was vortexed for 1 min. The reaction vessels were placed in an ultrasonic bath (Sonorex RK 510S) and digestion was finished within 30 min. For the destruction of residual HF, 1 mL of boric acid solution (4%, Merck, Germany), was added. Finally, the digestion solution still containing solids was centrifuged at 4000 rpm for 20 min.

Details on matrix

A01 - Dystric Cambisol (loamy sand, medium acid, very light humic) (tier 1) A06 - Cambic Rendzina (silty clay loam, very sub-acid, medium humic) (tier 1) A02 - Stagnic Luvisol (silt loam, sub-acid, light
humic) (tier 2) G03 - Eutric Cambisol (silt loam, medium acid, medium humic) (tier 2) G05 - Gleyic Fluvisol (silt loam, strongly acid, strongly humic) (tier 2) A01 - Dystric Cambisol (loamy sand, medium acid, very light humic) (tier 1) A06 - Cambic Rendzina (silty clay loam, very sub-acid, medium humic) (tier 1) A02 - Stagnic Luvisol (silt loam, sub-acid, light humic) (tier 2) G03 - Eutric Cambisol (silt loam, medium acid, medium humic) (tier 2) G05 - Gleyic Fluvisol (silt loam, strongly acid, strongly humic) (tier 2) These reference soils were also provided by the Fraunhofer Institute in Schmallenberg, Germany (www.refesol.de). Before use, all soils were air dried for 48 h at 21 °C and sieved by a 2 mm mesh. Analysis data of the used natural soils are given in Figure 1. The pH of soil A02 was 6.63 (CV 2.4%), for soil G03 5.64 (CV 1.2%) and 4.78 (CV 1.2%) for soil G05.

**Details on test conditions**

A defined soil / suspension mixture is shaken for a defined time. Afterwards the mixture is centrifuged to differentiate between adsorbed and non adsorbed material. Therefore the supernatant is analysed for its nanomaterial content. Based on the assumption that the nanomaterial not detected in the supernatant was adsorbed by the soil, the adsorbed amount is calculated. In a first study (tier 1) the soil / suspension ratio and shaking time was tested for tier 2. The adsorption by five different soils was analysed (tier 2). The test was run in duplicate The principal approach of the test is shown in Figure 2 Tier 1: Only used for P25. The soils A01 and A06 were tested with three different soil / suspension ratios (1/1, 1/5 and 1/25) and analysed after different equilibration time (4h, 8h, 24h, 48h) to determine the most suitable concentration for the actual adsorption test. Tier 2: Based on the results of the first tests with soil A01 and A06 a soil / suspension ratio of 1/5 and a contact time of 4 h was chosen for the following tests.

**Results and discussions**

**Results: Batch equilibrium or other method**

**Adsorption and desorption constants**

For all tested soils no significant concentrations of the nanomaterial was detected in the supernatant and no adsorption isotherms could be calculated.

**adsorption / desorption using a batch equilibrium method.**

![Figure 1: Sand, silt and clay content of the soils – A02 = Stagnic Luvisol, G03 = Eutric Cambisol and G05 = Gleyic Fluvisol (upper figure); cation exchange capacity (CEC), Ironoxalat (Feox), Aluminumoxalat (Alox) of the used soils (lower figure); (www.refesol.de).](image-url)
Overall remarks, attachments

Overall remarks
For the differentiation between adsorbed and non adsorbed particles by the soil matrix, the soil / suspension mixture was centrifuged to separate the solid from the aqueous phase. In contrast to soluble chemicals a loss of particles by centrifugation without adsorption on the soil matrix occur, for any particle size but mainly for larger particles. This can be important if agglomeration of the particles occurs, during the test. To derive first estimates for the settling behaviour of particles Stokes’ law can be used to calculate the velocity of small particles in a centrifugal field. For the above mentioned centrifuge with 2700 g all TiO2 particles larger than 177 nm settled and therefore are removed from the supernatant. It is conceivable that by contact of the TiO2 suspension with the soil / water mixture agglomeration of the particles occurred and due to this it is conceivable that during the centrifugation step the TiO2 agglomerates were separated from the liquid fraction without adsorption processes. The assumption that the amount of the materials which was not detected in the supernatant has to be absorbed by the soil is not true for nanomaterials. With the tested TiO2 materials no differentiation in the solid phase between settled and adsorbed particles is possible, which can lead to uncertain results. We conclude that the OECD test guideline 106 cannot be employed to derive information on adsorption – desorption isotherms at least for the nanomaterials tested here. Furthermore the test procedure itself is ambiguous in view of how any test results may be interpreted especially in view of separation of suspended nanomaterials along with soil particles by using a centrifuge or filter. Hence we recommend this test method not to be used for testing of nanomaterials.

Attached background material

diagramme_adsorption OECD 106_UV Titan M262.doc.docx
5.4.2 Henry's Law constant

5.4.3 Distribution modelling

5.4.4 Other distribution data

Endpoint study record: OECD312 by Institute of Energy and Environmental Technology (IUTA)

Administrative Data

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

Data source

Reference

Reference type study report
Author Carmen Nickel, Bryan Hellack, Stefan Gartiser, Felicitas Flach, Andreas Schiwy, Hanna Maes, Andreas Schäffer, Stephan Gabsch, Michael Stintz, Lothar Erdinger, Thomas A.J. Kuhlbusch
Year 2011
Title Fate and behaviour of TiO2 nanomaterials in the environment, influenced by their shape, size and surface area
Testing laboratory IUTA e.V., Duisburg, Hydrotox GmbH, Freiburg, RWTH Aachen, TU Dresden, Universitätssklinikum Heidelberg, Universität Duisburg-Essen, Germany
Report no. FKZ 3709 65 417

Materials and methods

Type of study soil leaching

Media
water - soil

Test guideline

Qualifier according to
Guideline OECD Guideline 312 (Leaching in Soil Columns)
Deviations

Test materials

Details on test material
Hydrophobic coated material. A part of the material lost his hydrophobic behaviour, after it was wetted with water. After suspension (using an ultrasonic homogenizer) the material lost his hydrophobic behavior \( \diamond \) indication that the coating of the material was affected by the suspension procedure. We hypothesized that the hydrophobic top layer of the coating (dimethicone) is washed off during suspension preparation.

Any other information on materials and methods incl. tables
Three different reference soiltypes were used: – A01 - Dystric Cambisol (loamy sand, medium acid, very light humic) A06 - Cambic Rendzina (silty clay loam, very sub-acid, medium humic) A04 - Gleyic
Podsol (loamy sand, medium acid, medium humic) The soitypes vary in their pH, texture and cationic exchange capacity. The reference soils were provided by the Fraunhofer Institute in Schmallenberg, Germany (www.refesol.de). Before use, all soils were air dried for 48 h at 21 °C and sieved through a 2 mm mesh. The soitype A01 showed a pH around 5.7, the soitype A06 a pH around 6.8 and soitype A04 a pH around 5.1. The test was run in duplicate under laboratory conditions, at a room temperature. • Exposure (e.g. powder or dispersion) 100 ml of a suspension with a concentration of 5 g/L TiO2 nanomaterial was applied on top of the soil columns. • Stock solutions preparation (vehicle, solvent, concentrations) and stability: A suspension with a concentration of 5 g/L was prepared. The average particle size in suspension after 10 min sonication using DLS measurements was 180 nm; n = 5. 500 mg of the Nanomaterial were weighted into a beaker glass, afterwards 100 ml DI water with a pH of 5 was added to the material. Afterwards the mixture was suspended by an ultrasonic homogeniser (Bandelin Sonoplus HD2200 ultrasonic homogeniser 200 W, Sonotrode VS70T) for 10 minutes. No additive was used. In DI water with a pH of 5 the suspensions showed a stable particle size distribution and zeta potential for 24 h (within a variance of 10 %). • Test temperature range: No specified conditions, the experiments were conducted under room conditions (15 – 25 °C) • Test design (number of replicates, concentrations): The test was run in duplicate with one reference system. 500 mg of the Nanomaterial were applied in 100ml suspension to the top of the soil column, afterwards a 0.01M CaCl2 solution (artifical rain) was applied for 48h. After leaching the columns were sectioned in four segments: - 1: top layer 0 - 1 cm, - 2: 3 - 4 cm, - 3: 16 - 17 cm, - 4: bottom layer 29 - 30 cm Each segment had a height of 1 cm. The segments were air dried, homogenised by grinding and chemically analysed. Representative samples were analysed with SEM and EDX. Chemical analysis The TiO2 concentration in the soil samples, the eluat as well as in the suspensions was analysed by ICP OES after digestion. Digestion Method Titantium dioxide in aqueous matrices is digested using a mixture of aqua regia and HF. Up to 10 mL of sample (depending on the type of sample) were pipetted into a PET test tube for centrifugation. Samples were concentrated to about 100 µL using a commercial concentration apparatus (Turbova, Zymark, Germany) at 70°C under a light nitrogen stream. After addition of aqua regia 2.4 mL HCl (36 - 38%, Ti < 0.2 ppb, J.T. Baker) and 0.8 mL HNO3 (69%, Ti < 0.5 ppb) 0.8 mL of HF (48%, Roth supra, Ti < 1 ppb) was added and the mixture was vortexed for 1 min. The reaction vessels were placed in an ultrasonic bath (Sonorex RK 510S) and digestion was finished within 30 min. For the destruction of residual HF, 1 mL of boric acid solution (4%, Merck, Germany), was added. Finally, the digestion solution still containing solids was centrifuged at 4000 rpm for 20 min. SEM EDX analysis Also from different segments of the soil column SEM EDX scan were conducted. Dynamic light scattering The particle size in suspension was measured by dynamic light scattering and also the zetapotential of the particles in suspension was analysed. Measuring procedure To gain reproducible measurements the dynamic light scattering measurements were standardised in this project. All chemical properties were shared between the project partners. For Malvern products a software internal SOP was generated for the measurement. The suspensions were sampled according to the recommendations of the DLS instruments manufacturers. Suspensions were sampled from the upper water column with a pipette without homogenisation.

Results and discussions
Remarks on results including tables and figures
Based on the quantitative ICP OES measurements a transport of the UV Titan M262 was indicated down to the fourth segment of the soil columns. Still, no Titanium above the detection limit was measured in the leachate of the columns. With SEM / EDX a transport of isolated TiO2 agglomerates was also detected in nearly all segments. All together it seems that soils do adsorb the main part of nanomaterials quite effectively or the high concentration employed lead to larger, more immobile agglomerates. Also a kind of clogging of the pores of the soil could have affected the transport. The latter may be of less importance since the water flow through the column was not significantly affected. It is conceivable that the coating of the UV Titan M262 (aluminiumoxide and dimethicone) could have affected the transport.
behaviour in the tested soil systems. No specific recommendation can be given with regard to the way the nanomaterial should be brought onto the soil column. We decided to follow a likely path of environmental entry by using a suspension, which also allows for reproducible conditions in view of particle sizes. Still a premixing of the nanomaterial with a soil section may also be used. Overall the OECD Method 312 can be used for the testing of nanomaterials. Still, a clear definition of exposure scenarios should be given to mimic more realistic concentrations and avoid different findings due to different concentrations employed. Therefore, specific analytical tools may have to be developed to allow simulations at lower concentrations. At least for TiO2 the analysis is a challenge in view of the soil background concentration.

Figure 37: Soil column run with UV Titan M252 and soil A01 Dystric Cambisol. Natural Ti in soil A01 0.19% (1.9 g/kg). Error bars = max and min, n = 2.
Figure 39. SEM/EDX scans of segment one of soil A01 treated with UV Titan M262. The lower right scan shows the negative control with no Ti detected (scan 4).
Figure 40: SEM/EDX scans of segment four of soil A01 treated with UV Titan M262. The right scan shows the negative control with no Ti detected (scan 2).

Figure 41: Soil column run with UV Titan M262 and soil A06 Cambic Rendzina. Ti background concentration of soil A06 was 0.42% (4.2 g/kg). Error bars = max and min; n = 2.
Overall remarks, attachments

Overall remarks
In the test guideline several criteria for soil selection are provided with regard to pH values, texture and organic content. But no limitations concerning their water permeability are made. In this study a backwater soil (Gleyic Podsol A04) was used whereupon difficulties occurred due to the lack of breakthrough by gravity for water. A once applied suction power overcame this problem and triggered a run off of the material. Unluckily the transport was mainly along the glass column wall, as could be seen by the deposition of TiO2 along the column, which in turn made a statement about the mobility of a material in soils difficult. • We recommend limiting the use to soil types with normal to low retention potential in soil column tests which allow water transport by gravity. If other soil types are employed, needing the aid of suction power, all data should be carefully evaluated for transport mainly along the soil column walls. Concentration and detection • We recommend the test scenario to be clearly defined for better comparison between different nanomaterials. In the here chosen case of TiO2 relatively high, worst case scenario concentrations had to be employed possibly leading to high agglomeration and pore clogging. If another nanomaterial with significantly lower concentration is used possibly a higher transport rate could be determined. Hence information on concentration dependent effects in soil column test for nanomaterials is needed • We recommend that for the simulation of a more realistic scenario the test design should be adapted for the application of lower concentrated suspensions over a longer time period. • We recommand that the sampling of different alliquots of the eluate can be necessary, to achieve a higher concentration in the sample. • We recommend some detailed tests using also other detection methods in the eluate, like Field Flow Fractionation coupled with a mass spectrometer (FFF-MS) or Surface-Enhanced Raman Spectroscopy (SERS) which are able to detect nanomaterials in matrices with a high background of other materials. However these detection methods are expensive and the detailed studies should be conducted mainly for method evaluation purposes. More information about
detection methods can be found in Tiede et al., 2008. • SEM / EDX analysis is a useful tool to detect the transport of isolated TiO2 agglomerates and tiny amounts of Ti and their shape. Chemical analysis The use of an ICP MS was not possible because interferences occurred with the used acids for the digestion; therefore ICP OES was used for quantitative chemical analysis.

Attached background material
figures_OECD 312_M262.docx
Attached document 4: SOP ICP OES Al and Si in solid samples.pdf:

5.6 Other relevant information

6. ECOTOXICOLOGICAL INFORMATION

6.1 Aquatic toxicity

6.1.1 Short-term toxicity to fish

*Endpoint study record: zebrafish 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**

**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 103, Titanium Dioxide (calcined) approx.89 % Modification Rutile BET surface area 60 m²/g

**Details on sampling**
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
Zebra fish, Danio rerio, embryos obtained in own facilities Age at study initiation: 2 h after spawning
Control group: received only embryo medium

Study design

Test type
static

Total exposure duration
8 d

Remarks

Test conditions

Test temperature
28.5 °C

Nominal and measured concentrations
10, 100 and 1000 mg/l

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. 10 embryos exposed to 10, 100 and 1000 mg/l of each TiO2 NM. two replicated per treatment and NM

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
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.

*Acute Toxicity to Fish

Department 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>
<thead>
<tr>
<th>TREATMENT</th>
<th>Exp(mg/l)</th>
<th>HATCHED</th>
<th>DEAD</th>
<th>SURVIVORS</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>17.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM 101</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 A</td>
<td>7</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 B</td>
<td>6</td>
<td>13.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 A</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 B</td>
<td>7</td>
<td>15.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 A</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 B</td>
<td>7</td>
<td>16.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM 103</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 A</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 B</td>
<td>8</td>
<td>14.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 B</td>
<td>9</td>
<td>1</td>
<td>8.00</td>
<td>88.89</td>
<td></td>
</tr>
<tr>
<td>1000 A</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 B</td>
<td>6</td>
<td>21.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM 104</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 A</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 B</td>
<td>8</td>
<td>16.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 A</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 B</td>
<td>7</td>
<td>15.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 A</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 B</td>
<td>2</td>
<td>10.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM 105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 A</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 B</td>
<td>8</td>
<td>14.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 A</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 B</td>
<td>9</td>
<td>18.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 A</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 B</td>
<td>8</td>
<td>15.00</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-25 Evonik</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 A</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
<td>----</td>
<td>---</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td><strong>10 B</strong></td>
<td>9</td>
<td></td>
<td>18.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>100 A</strong></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>100 B</strong></td>
<td>5</td>
<td></td>
<td>13.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>1000 A</strong></td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1000 B</strong></td>
<td>10</td>
<td></td>
<td>19.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>P-25 Evonik</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>10 A</strong></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>10 B</strong></td>
<td>9</td>
<td></td>
<td>16.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>100 A</strong></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>100 B</strong></td>
<td>7</td>
<td></td>
<td>15.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>1000 A</strong></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1000 B</strong></td>
<td>8</td>
<td>1</td>
<td>14.00</td>
<td>93.33</td>
<td></td>
</tr>
<tr>
<td><strong>P-25 Evonik</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>10 A</strong></td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>10 B</strong></td>
<td>8</td>
<td></td>
<td>17.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>100 A</strong></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>100 B</strong></td>
<td>8</td>
<td></td>
<td>16.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>1000 A</strong></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1000 B</strong></td>
<td>8</td>
<td></td>
<td>16.00</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
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.

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: 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, Spian

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 103, Titanium Dioxide (calcined) approx.89 % Modification Rutile BET surface area 60 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.

Test organisms
Test organisms (species)
Daphnia magna

Details on test organisms
Neonates < 24 day old

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

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
14:10 h light: dark, 400 lux without food

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. Mortality was negligible < 10% in all tested exposure levels, thus it was not possible to
estimate any LC50 < 1.5 g/l. The percentage of immobile individuals was used as endpoint, transformed into probits and used to determine LC50s (Finey 1971).

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

**Guideline** OECD Guideline 211 (Daphnia magna Reproduction Test)

**Deviations**

Test materials

**Details on test material**

NM 103, Titanium Dioxide (calcined) approx. 89% Modification Rutile BET surface area 60 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 ºC 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 ºC
**pH**
7.8 to 8.1

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

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

**Nominal and measured concentrations**
0, 1, 3, 10 mg/L

**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**

<table>
<thead>
<tr>
<th>Duration</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoint</td>
<td>LOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>&gt; 10 mg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
<tr>
<td>Conc. based on</td>
<td>reproduction</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>21 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>&gt; 10 mg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
<tr>
<td>Conc. based on</td>
<td></td>
</tr>
</tbody>
</table>
### Details on results
Mortality was negligible during tests. 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. Reproduction: In Exp 1 total offspring production was lower than control treatment (LOEC); NM 103 at 1 mg/L and NM 8 at 10 mg/L. However, since in NM 103 the effect was not dependent on the dose and at higher doses no effect was observed the LOEC and NOEC was established as higher than 10 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

<table>
<thead>
<tr>
<th>Basis for effect</th>
<th>reproduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>21 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>LOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>10 mg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
<tr>
<td>Conc. based on</td>
<td></td>
</tr>
<tr>
<td>Basis for effect</td>
<td>other: population growth rate</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>21 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>NOEC</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>3 mg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>nominal</td>
</tr>
<tr>
<td>Conc. based on</td>
<td></td>
</tr>
<tr>
<td>Basis for effect</td>
<td>other: population growth rate</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td></td>
</tr>
</tbody>
</table>
B. Chronic Toxicity to Aquatic Invertebrates

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

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, 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 80oC 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 monodispered 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 80oC. 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 22oC ± 1 oC with 16h light and 8h dark photoperiod. TetraminTM flakes (Tetra Werke, Blacksburg, VA, U.S.A.) were ground up and passed through 500μm sieve, organisms received 5mg of dry TetraminTM 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 22oC ± 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 TetraminTM 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

<table>
<thead>
<tr>
<th>Duration</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endpoint</td>
<td>IC50</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>36.4 mg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>meas. (not specified)</td>
</tr>
<tr>
<td>Conc. based on</td>
<td>growth dry weight</td>
</tr>
<tr>
<td>Basis for effect</td>
<td>Remarks (e.g. 95% CL)</td>
</tr>
<tr>
<td>Duration</td>
<td>28 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>other: IC20</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>5.5 mg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>meas. (not specified)</td>
</tr>
<tr>
<td>Conc. based on</td>
<td>Basis for effect</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td>NM103</td>
</tr>
<tr>
<td>Duration</td>
<td>28 d</td>
</tr>
<tr>
<td>Endpoint</td>
<td>LC50</td>
</tr>
<tr>
<td>Effect conc.</td>
<td>1404 µg/L</td>
</tr>
<tr>
<td>Nominal/Measured</td>
<td>meas. (not specified)</td>
</tr>
<tr>
<td>Conc. based on</td>
<td>Basis for effect</td>
</tr>
<tr>
<td>Remarks (e.g. 95% CL)</td>
<td>Duration</td>
</tr>
</tbody>
</table>
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 were chronically (28d) exposed to sonicated mixtures of NM103 TiO2 NPs which have a slightly hydrophobic surface by treatment with dimethicone (2%). Exposure to nominal concentration of 20, 50, and 100 mg TiO2 /L which correspond to measured concentration of 6.6 ± 1.1, 19.6 ± 1.1, and 53.7 ± 6.2 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. An IC50 value of 36.4 ± 2.8 mg TiO2 /L and an IC20 value of 5.5 ± 0.8 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
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: in vitro tadpole by University of Victoria

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 University of Victoria, Canada
Cross-reference to same study

Materials and methods

Principles of method if other than guideline
C-fin Assay Ex vivo – Organ culture

Test materials

Details on test material
in 70% L-15 medium (C-Fin culture medium): DLS effective diameter of non-sonicated M262 at 80 mg/L with 0.1% (v/v) DMSO: 1043.5nm in dH2O: DLS effective diameter of non-sonicated M262 at 80 mg/L with 0.1% (v/v) DMSO: 461.13nm agglomeration/ aggregation: significant, although effect of sonication (which was performed during exposures) was not determined Zeta-Potential: in 70% L-15 medium (C-Fin culture medium): Zeta potential of non-sonicated M262 at 80 mg/L with 0.1% (v/v) DMSO: +2.16mV in dH2O: Zeta potential of non-sonicated M262 at 80 mg/L with 0.1% (v/v) DMSO: +36.89mV

Details on sampling
Dispersed in DMSO, final concentration: 0.1% (v/v) 70% Leibovitz-15 culture medium (Gibco, Invitrogen # 41300-039) with 10% HEPES (Sigma # H4034-500G) with Penicillin and Streptomycin (Invitrogen #15140-122) and l-glutamine (Sigma #G7513) Dispersion/sample preparation protocol (e.g. concentration, duration, method, energy input): Powder added to DMSO and vortexed to disperse Diluted to 1000x test concentrations in DMSO and sonicated for 10min on low with 30 sec on/off cycle (Bioruptor ECD200, Diagenode Inc., Sparta NJ, USA) Diluted 1/1000 into 70% L-15 culture medium and vortexed to disperse

Test organisms

Test organisms (species)
Rana catesbeiana

Details on test organisms
cultured tailfin biopsies from premetamorphic TK2 VI-VIII Bullfrog Tadpoles

Study design

Total exposure duration
48 h

Remarks

Test conditions

Test temperature
25°C

Nominal and measured concentrations
8-800 ng/L TiO2; ± 10 nM TH
Details on test conditions

In brief: Eight 6 mm biopsies were collected from each of eight *Rana catesbiana* tadpoles, from the dorsal and ventral tail fins. Each biopsy was put into a different treatment, and therefore all animals were represented in each treatment. Eight treatments included a negative control and 3 increasing doses of TiO$_2$ ± thyroid hormone (TH). NaOH is the vehicle for TH and was therefore included in all treatments. 1000x stocks of TiO$_2$ were prepared in DMSO for each dose, sonicated and diluted to 1x into the L-15 culture medium. Biopsies were cultured in 1ml of each treatment for 48hrs, with a 2hr pre-incubation in TiO$_2$ before addition of TH (in NaOH) or NaOH. Biopsies were subsequently preserved in RNAlater until RNA was extracted, then cDNA was generated via reverse transcription for analysis with real-time quantitative polymerase chain reaction (QPCR). The steady-state level of mRNA abundance was determined for thyroid hormone responsive genes [thyroid hormone receptor alpha and beta (TRa, TRb, respectively); *Rana* larval keratin 1 (RLK1)] and stress-responsive genes [catalase (CAT); heat-shock protein 30 (Hsp30); super-oxide dismutase (SOD)], as well as the non-variant ribosomal protein L8 (rpL8). Test design: Dose response and TH challenge to determine deviations in response to TH Number of replica: 8 biopsies from each of 8 animals therefore each treatment has biopsies from the same 8 animals Frequency of Dosing: 2 hr pre-incubation in TiO$_2$ containing medium before addition of NaOH or TH in NaOH Positive and negative control groups and treatment negative control for TiO$_2$ (no TH): NaOH and DMSO negative control for TiO$_2$ + TH: TH in NaOH and DMSO Solvent: for TiO$_2$: DMSO for TH: NaOH in dH$_2$O Criteria for evaluating results: Transcriptional perturbation of genes involved in Thyroid hormone signaling (TRa, TRb, RLK1) or cellular stress (CAT, Hsp30, SOD).

Any other information on materials and methods incl. tables

in the dark

Results and discussions

Details on results

At 800 ng/L TiO$_2$, expression of TRb was decreased by 1.2-fold in the absence of TH (p-value = 0.022). At 80 ng/L TRa was decreased by 1.3-fold in the presence of TH (p-value = 0.020). While not statistically significant, 80 ng/L TiO$_2$ with TH also elevated steady-state levels of Hsp30 transcripts 3.3-fold (p = 0.054). At 800 ng/L TiO$_2$, expression of TRb was decreased by 1.2-fold in the absence of TH (p-value = 0.022). At 80 ng/L TRa was decreased by 1.3-fold in the presence of TH (p-value = 0.020). While not statistically significant, 80 ng/L TiO$_2$ with TH also elevated steady-state levels of Hsp30 transcripts 3.3-fold (p = 0.054).

Reported statistics and error estimates

Since the eight biopsies from each animal were in each of the eight treatments, samples were not independent and Pairwise Friedman test for repeated measures was performed. Statistical significance was considered at p-value < 0.05

Applicant's summary and conclusion

Conclusions

At 800 ng/L TiO$_2$, expression of TRb was decreased by 1.2-fold in the absence of TH (p-value = 0.022). At 80 ng/L TRa was decreased by 1.3-fold in the presence of TH (p-value = 0.020). While not statistically significant, 80 ng/L TiO$_2$ with TH also elevated steady-state levels of Hsp30 transcripts 3.3-fold (p = 0.054).

Cross-reference to other study


6.2 Sediment toxicity

6.3 Terrestrial toxicity

6.3.1 Toxicity to soil macroorganisms except arthropods

Endpoint study record: Earthworm reproduction.001 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 1st test: April 7 - June 2, 2010; 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 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 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 identity
Identifier
Identity NM-103
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.

Vehicle

no

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 was the direct introduction of TiO2 into the earthworm feed, which consisted of antibiotics-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 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 maximum water holding capacity
Nominal and measured concentrations
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 NM 103: 50, 100, 200, 400 mg/kg soil, dry mass (application via powder on soil).

Details on test conditions
The soil used in the test was a natural sandy soil (certified RefeSol 01-A, batch IME 01: sand 71%, silt: 24%, clay: 5%, Org C: 0.93%, pH 5.7, clay: 5%). The soil was sieved to ≤ 2 mm. The soil was not sterilized and had been stored outdoors in high-grade stainless steel basins with drainage and ground contact at the test facility. 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 1: TiO2: Incubation conditions in the reproduction test with earthworms NM 103 first test NM 103 second test Incubation temperature [°C] 19 – 21 19 – 21 Light intensity [lx] 600 – 750 600 – 800 Soil dry mass [%] 80 - 90 81 – 88 pH (1 mol/L KCl) – test start 4.9 - 5.0 5.0 pH (1 mol/L KCl) – test end 6.2 – 6.6. 6.8 – 6.9

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 All 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 con¬tainers 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;= 400 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>2nd test; application via powder on soil; application via dispersion on feed and soil and via powder on feed and soil (1st test) showed no effect up to the highest test concentration (200 mg/kg for powder application and 20 mg/kg via dispersion)</td>
</tr>
</tbody>
</table>

Details on results

All raw data are included in the attached document.

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.

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.
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

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. In some of the tests the Ti concentration in earthworms was determined. 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. In some of the tests the Ti concentration in earthworms was determined. 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.
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: by Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM)*

Administrative Data

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

Data source

Reference
Reference type  study report
Author  Otto Creutzenberg
Year  2013
Title  Toxic Effects of Various Modifications of a Nanoparticle Following Inhalation
Bibliographic source  BAuA Research Project F 2246
Testing laboratory  Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM) Nikolai Fuchs Strasse 1 30625 Hannover, Germany
Report no.  Fraunhofer ITEM Study No. 02 N 11 538
Owner company  Federal Institute for Occupational Safety and Health

Materials and methods

Type of method
in vivo
Test guideline

Qualifier according to
Guideline other guideline: OECD 412
Deviations yes 90 days post exposure

Test materials

Details on test material
Unfortunately, the investigators misidentified the NM103 and NM104 test samples. In the Fraunhofer report, NM103 is identified in table 3.1 on page 32 as “hydrophobic...UV TITAN M212 – rutile nanoTiO2” while NM104 is identified as “hydrophilic...UV TITAN M262 rutile nano TiO2.” In contrast to this identification in the Fraunhofer report, NM104 is “UV TITAN M212”, and NM103 is “UV TITAN M262”. Thus, the conclusions in the reported for the NM104 may really apply to NM103, and vice versa.

Test animals

Species rat

Strain Wistar

Sex male

Details on test animals and environmental conditions
Male Wistar rats [strain Crl:WI (Han)] were purchased from Charles River Deutschland (Sulzfeld, Germany). The age of the animals at the start of exposure was approx. 8 weeks and the weight approx. 270 gram. Rats were exposed to the test item by nose-only inhalation. For a period of 2 - 3 weeks prior to exposure animals were trained to become accustomed to nose-only tubes.

Administration / exposure

Route of administration inhalation: aerosol

Details on exposure
Rats were exposed to aerosol concentrations (low, mid, high) of 3, 12 and 48 mg/m\textsuperscript{3} for 28 days (6 hours/day, 5 days/week) while concurrent controls inhaled clean air. This dosing scheme was aiming at achieving non-overload, partial overload and complete overload conditions in the low, mid and high dose groups, respectively. Subsequently, endpoints were analysed at day 3, 45 and 94 of the post-exposure period. Calculation: According to the MPPD model the deposition rate for particles with an agglomerate density $\rho_{\text{Agg}}$ = approx. 1.7 and an MMAD of approx. 1 $\mu$m amounts to approx. 7%. In a 28-day test the retained particles masses would result in approx. 0.20, 1.0 and 6 mg/lung, respectively.

Control animals
yes
**Details on study design**

All animals were subjected to a complete necropsy, which included a careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. For anesthesia an overdose of carbon dioxide was used. The abdominal cavity was opened and the diaphragm was cut carefully allowing the lungs to collapse. Heart, esophagus, upper half of trachea, thymus and lung associated lymph nodes (LALN) were removed from the lung convolution. The lung was inflated under a pressure of about 20 cm water with formalin and was fixed by immersion for a minimum of 2 hours, and used for histopathology. Thereafter the weight of the lower part of the trachea was recorded and the weight of the lung was calculated. The following organs were trimmed and wet weights were recorded: liver, kidneys, testes, epididymides, thymus, spleen, brain, and heart. All tissues listed in OECD Guideline no. 412, table 2 were prepared for histopathology. The trimming was done according to Ruehl-Fehlert et al. (2003), Kittel et al. (2004) and Morawietz et al. (2004). The following histopathology was performed in 6 animals per group after end of exposure (day 3) and in the recovery group animals on day 45 and day 94 after exposure:  
- full histopathology on the respiratory tract and other organs and tissues, as listed in OECD 412 of all animals in the clean air control group and the high dose groups and all animals that died or were killed during the study.  
- histopathology of the left lung lobe, including bronchi and the lung-associated lymph nodes (LALN), trachea, larynx, pharynx, the nasal cavities (turbinales) and visceral pleura in all animals of all groups. Lungs were fixed in buffered formalin (10%), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). A special stain was applied for diagnosis of fibrotic changes: Masson trichrome. After sacrifice the right lung lobes were used for transmission electron microscopy (TEM) analysis. Transmission Electron Microscopy (TEM) Analysis:  
At the respective time points to be investigated (recovery day 3, 45 and 94) the right part of the lung including the right cranial, right middle and right caudal lung lobe as well as the accessory lung lobe were fixed by instillation using 5% glutaraldehyde solution (pH 7.2) for at least 24 hours. Following fixation of the tissue, the volume of the fixed tissue was determined using the method of Scherle (1970). The basic principal of this method is that the displacement of water equals the volume of the object. The displaced water leads to an increased weight which can be measured. The volume of the lung tissue was measured to detect a possible age- or treatment-dependent affect on the lung volume which would influence the measurement of the particle amount in the transmission electron microscope. Thereafter, to avoid biased sampling, multiple specimens per organ were taken using the systematic uniform random sampling method. These samples were postfixed in 1% osmiumtetroxid in 0.1M sodium cacodylate buffer. Following dehydration of the tissue in a graded series of ethanol the specimens were infiltrated with epoxy resin and embedded. An ultramicrotome (Ultracut E, Richard-Jung) was used for cutting ultrathin sections (70nm) of three randomly chosen samples per animal. They were positioned on copper grids and observed with a transmission electron microscope (Zeiss, Leo 910). 55000 square micrometer of each sample were investigated at a magnification of 10.000x. The amount and location of the nanoparticles found was noted and assigned to compartments. The compartments in which nanoparticles were detected were defined as follows: intraalveolar macrophage, free within the alveolus, pneumocyte type II, free within the interstitium, interstitial macrophage, interstitial cellular (not otherwise definable), bronchiolar epithelium. For statistical analysis of the transmission electron microscopy results the software Statistica (Statsoft, USA) was used and an analysis of variance was applied.

**Statistics**

Differences between groups were considered statistically significant at p < 0.05. Data were analyzed using analysis of variance. If the group means differed significantly by the analysis of variance the means of the treated groups were compared with the means of the control groups using Dunnett's test. The statistical evaluation of the histopathological findings will be done with the twotailed Fisher test by the PROVANTIS system. If necessary, further statistical procedures will be applied upon agreement with the sponsor.
Overall remarks, attachments

Overall remarks
for results see attachment

Attached full study report
Attached document: 02N11358_300912_final_090413.pdf (general annex):
ENV/JM/MONO(2015)17/ANN21

**Endpoint study record: Basic toxicokinetics_NM 103_ IV 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 type | study report |
| Author | W De Jong |
| Year | 2013 |
| Title | Deliverable 7: Identification of target organs and biodistribution including ADME parameters |

| Bibliographic source | Testing laboratory | RIVM (NL) | Report no. | D7 |
| Owner company |
| Company study no. | Report date |

**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**

| Identifier | Reference Material/Nanomaterial |
| Identity | NM 103 |
Test materials
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Test animals
Species
rat

Strain
Wistar

Sex
male/female

Administration / exposure
Route of administration
intravenous

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: Single (day 1) or repeated (on 5 consecutive days, day 1-5) Sampling time: - Single admin: day 2 and day 90 - Repeated admin: day 6, 14, 30 and 90

Doses / concentrations
2.3 mg of TiO2 resulting in a dose of 8.7-9.7 mg/kg bw/d (male) and 12.4-13.7 mg/kg bw/d (female) 5 day cumulative dose: 43.5-48.5 mg/kg bw (male) and 62-68.5 mg/kg bw (female)

No. of animals per sex per dose
Treated Groups: 3 M + 3 F except at day 14 and day 30: 3 MControl: vehicle 2 M + 1 F for day 2, 6, 90. No control at day 14 and 30

Control animals
yes

Details on dosing and sampling
Sampling: liver, spleen, kidneys, thymus, heart, lungs, lymph nodes (mesenteric and popliteal), brain, bone including bone marrow (femur), testes/ovaries, skin, muscle

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 porocol in annex
Applicant's summary and conclusion

Interpretation of results

other: Major target organs: liver (29-64%) > spleen (1-10%) > lung > kidney
Rapid distribution from the bloodstream to the organs
Slow to no excretion in urine and feces

Conclusions

TiO2 NM 103 is rapidly distributed the bloodstream to the organs with liver > spleen lung > kidney and
remain stored in the body for a period of 90 at least.

Cross-reference to other study


Endpoint study record: Basic toxicokinetics_NM_103_Gavage 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>W De Jong</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 7: Identification of target organs and biodistribution including ADME parameters</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>NRCWE (DK)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D.7</td>
</tr>
<tr>
<td>Owner company</td>
<td></td>
</tr>
<tr>
<td>Company study no.</td>
<td>Report date</td>
</tr>
</tbody>
</table>

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
Identifier    Reference Material/Nanomaterial
Identity      NM 103

Test materials
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

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 (male) and 13.1-15.2 mg/kg bw (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

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

Overall remarks, attachments
Attached full study report
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 porocol in annex
Applicant's summary and conclusion

Interpretation of results

no bioaccumulation potential based on study results Level of Ti close to the limit of detection or below the limit of detection in the liver and spleen. Only incidentally some TiO2 could be detected at or above the limit of detection in these organs. No uptake in the lymphnodes

Conclusions

No evidence for uptake of NM-103 following short term exposure via gavage

Cross-reference to other study

7.2 Acute Toxicity

7.2.1 Acute toxicity: oral

7.2.2 Acute toxicity: inhalation

Endpoint study record: Intratracheal instillation by Fraunhofer Institute for Toxicology and Experimental Medicine (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)

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
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

7.5.1 Repeated dose toxicity: oral
7.5.2 Repeated dose toxicity: inhalation

Endpoint study record: Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM)

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

Data source
Reference
Reference type study report
Author Otto Creutzenberg
Year 2013
Title Toxic Effects of Various Modifications of a Nanoparticle Following Inhalation
Bibliographic source BAuA Research Project F 2246
Testing laboratory Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM) Nikolai Fuchs Strasse 1 30625 Hannover, Germany
Report no. Fraunhofer ITEM Study No. 02 N 11 538
Owner company Federal Institute for Occupational Safety and Health

Materials and methods
Test guideline
Qualifier according to
Guideline OECD Guideline 412 (Repeated Dose Inhalation Toxicity: 28/14-Day)
Deviations yes 90d postexposure study

Test animals
Species rat
Strain Wistar
Sex male
Details on test animals and environmental conditions
Male Wistar rats [strain Crl:WI (Han)] were purchased from Charles River Deutschland (Sulzfeld, Germany). The age of the animals at the start of exposure was approx. 8 weeks and the weight approx. 270 gram. Rats were exposed to the test item by nose-only inhalation. For a period of 2 - 3 weeks prior to exposure animals were trained to become accustomed to nose-only tubes.

Administration / exposure
Route of administration
inhalation: aerosol

Type of inhalation exposure
nose only

Duration of treatment / exposure
Rats were exposed to aerosol concentrations (low, mid, high) of 3, 12 and 48 mg/m3 for 28 days (6 hours/day, 5 days/week) while concurrent controls inhaled clean air. This dosing scheme was aiming at achieving non-overload, partial overload and complete overload conditions in the low, mid and high dose groups, respectively. Subsequently, endpoints were analysed at day 3, 45 and 94 of the post-exposure period. Calculation: According to the MPPD model the deposition rate for particles with an agglomerate density ρAgg = approx. 1.7 and an MMAD of approx. 1 μm amounts to approx. 7%. In a 28-day test the retained particles masses would result in approx. 0.20, 1.0 and 6 mg/lung, respectively.

Details on study design
The particulate sample aerosols were generated by dry dispersion with pressurized air. Dispersion was achieved by a feeding system and a high-pressure, high-velocity pressurized air dispersion nozzle developed by Fraunhofer ITEM (Koch, 1998). For each nose-only exposure unit, the aerosol was generated by a high-pressure pneumatic disperser. The disperser was fed with the test/reference items under computerized control, i.e. with a feed back loop to the actual aerosol concentrations measured by an aerosol photometer (see Figure 3.3). The photometer gives a scattering light signal which is proportional to the particle concentration, if the particle size distribution is constant. The ratio between photometer signal and concentration was determined throughout the study by comparing to gravimetric concentrations. The aerosol was given to the rats by a flow-past nose-only inhalation exposure system which was used for previous particle and fiber inhalation studies at Fraunhofer ITEM. In this system, aerosols were supplied to each rat individually, and exhaled air was immediately exhausted. The airflow to each rat was approximately 1 l/min which is calculated to be laminar. Therefore measurement of the oxygen concentration is not necessary. Prior to the 28-day exposure of rats, technical trials to adjust particle size distributions and exposure levels were conducted. Additionally, the mass median aerodynamic diameter (MMAD) was determined 2-3 times using a cascade impactor (Marple impactor). Filter samples of the aerosols were taken daily to control the aerosol concentrations and to calibrate the aerosol photometers. These samples were collected at a port of the nose-only exposure unit, thus, under the same conditions the rats are inhaling the aerosol. In Table 3.3, the means of the aerosol concentrations are summarized comprising the exposure period from September 19 to October 18, 2011. Each animal was exposed for 20 days. The means are close to the target concentrations.

Examinations
Sacrifice and pathology
All animals were subjected to a complete necropsy, which included a careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. For anesthesia an overdose of carbon dioxide was used. The abdominal cavity was opened and the diaphragm was cut carefully allowing the lungs to collapse. Heart, esophagus, upper half of trachea, thymus and lung
associated lymph nodes (LALN) were removed from the lung convolution. The lung was inflated under a pressure of about 20 cm water with formalin and was fixed by immersion for a minimum of 2 hours, and used for histopathology. Thereafter the weight of the lower part of the trachea was recorded and the weight of the lung was calculated. The following organs were trimmed and wet weights were recorded: liver, kidneys, adrenals, testes, epididymides, thymus, spleen, brain, and heart. All tissues listed in OECD Guideline no. 412, table 2 were prepared for histopathology. The trimming was done according to Ruehl-Fehlert et al. (2003), Kittel et al. (2004) and Morawietz et al. (2004).

Other examinations
The following histopathology was performed in 6 animals per group after end of exposure (day 3) and in the recovery group animals on day 45 and day 94 after exposure: · full histopathology on the respiratory tract and other organs and tissues, as listed in OECD 412 of all animals in the clean air control group and the high dose groups and all animals that died or were killed during the study. · histopathology of the left lung lobe, including bronchi and the lung-associated lymph nodes (LALN), trachea, larynx, pharynx, the nasal cavities (turbinales) and visceral pleura in all animals of all groups. Lungs were fixed in buffered formalin (10%), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). A special stain was applied for diagnosis of fibrotic changes: Masson trichrome. After sacrifice the right lung lobes were used for transmission electron microscopy (TEM) analysis.

Statistics
Differences between groups were considered statistically significant at p < 0.05. Data were analyzed using analysis of variance. If the group means differed significantly by the analysis of variance the means of the treated groups were compared with the means of the control groups using Dunnett's test. The statistical evaluation of the histopathological findings will be done with the twotailed Fisher test by the PROVANTIS system. If necessary, further statistical procedures will be applied upon agreement with the sponsor.

Results and discussions
Observations
Clinical signs and mortality
no effects

Body weight and weight gain
no effects

Food consumption
not examined

Food efficiency
not examined

Water consumption
not examined

Ophthalmoscopic examination
not examined

Haematology
yes
Clinical chemistry
yes

Urinalysis
not examined

Neurobehaviour
not examined

Organ weights
yes

Gross pathology
yes

Histopathology: non-neoplastic
yes

Histopathology: neoplastic
not examined

Details on results
see attachment

Overall remarks, attachments
Attached full study report

7.5.3 Repeated dose toxicity: dermal
7.5.4 Repeated dose toxicity: other routes
7.6 Genetic toxicity

7.6.1 Genetic toxicity in vitro

Endpoint study record: Genetic toxicity in vitro_NM 103_COMET 3D skin 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>BfR (GER)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D5</td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed yes, but willing to share

Materials and methods

Type of genotoxicity
DNA damage and/or repair

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

Test guideline

Qualifier no guideline available

Guideline

Deviations

Test materials

Test material equivalent to submission substance identity yes
Reference Material/Nanomaterial and Sample identification number

**Identifier**  Reference Material/Nanomaterial  
**Identity**  NM 103

Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

**Method**

**Species/strain**

<table>
<thead>
<tr>
<th>Details on mammalian cell lines (if applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additional strain characteristics</td>
</tr>
</tbody>
</table>

**Metabolic activation**

**Metabolic activation system**

**Test concentrations**

82/164/246 µg/cm²

**Details on test system and conditions**

single dose with incubation time of 72h

**Evaluation criteria**

percentage of DNA in the tail (% Tail DNA) with 200 cells scored per dose

**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 detailed protocol in annex

**Applicant's summary and conclusion**

**Interpretation of results**

negative

**Conclusions**

TiO₂ NM 103 does not induce DNA strand breaks in human reconstructed skin model following 72h treatment with the alkaline comet assay.

**Cross-reference to other study**

Endpoint study record: Genetic toxicity in vitro_NM_103_COMET 16-HBE 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bibliographic source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testing laboratory</td>
</tr>
<tr>
<td>Report no.</td>
</tr>
</tbody>
</table>

| Owner company       |
| Company study no.   |

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Type of genotoxicity
DNA damage and/or repair

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

Test guideline

Qualifier no guideline available

Guideline

Deviations

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM 103
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Method
Species/strain
Species/strain mammalian cell line, other:
Details on mammalian cell lines (if applicable) human bronchial epithelial 16 HBE cells
Additional strain characteristics
Metabolic activation
Metabolic activation system

Test concentrations
2/8/32/128/512 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time of 3 h and 24 h

Evaluation criteria
percentage of DNA in the tail (% Tail DNA) with 300 cells scored per dose

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 detailed protocol in annex

 Applicant's summary and conclusion

Interpretation of results
negative

Conclusions
TiO2 NM 103 does not induce DNA strand breaks in 16-HBE cells following both 3h and 24 h incubation with the alkaline comet assay.

Cross-reference to other study

Endpoint study record: Genetic toxicity in vitro_NM 103_COMET A549 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**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>study report</td>
<td>H Norppa</td>
<td>2013</td>
</tr>
</tbody>
</table>

**Title**
Deliverable 5: In vitro testing strategy for nanomaterials including database

**Testing laboratory**
NIOM (PL)

**Report no.**
D5

**Company study no.**

### Data access

other: Owner: NANOGENOTOX

**Data protection claimed**
yes, but willing to share

### Materials and methods

**Type of genotoxicity**
DNA damage and/or repair

**Type of study**
single cell gel/comet assay in mammalian cells for detection of DNA damage

**Test guideline**
- **Qualifier**
  no guideline available
- **Guideline**
- **Deviations**

**Test materials**

**Test material equivalent to submission substance identity**

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

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Material/Nanomaterial</td>
<td>NM 103</td>
</tr>
</tbody>
</table>

**Details on test material**

Commercial name: UV TITAN M262 (Sachtleben)

**Method**

**Species/strain**

- mammalian cell line, other:
  - human alveolar epithelial A549 cells

**Additional strain characteristics**
Metabolic activation

Test concentrations
50/100/256 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time of 3 h and 24 h

Evaluation criteria
percentage of DNA in the tail (% Tail DNA) with 200 cells scored

Statistics
ANOVA test with Dunnett’s post-hoc test

Overall remarks, attachments
Attached full study report 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 detailed protocol in annex

Applicant's summary and conclusion
Interpretation of results
negative

Conclusions
TiO2 NM 103 does not induce DNA strand breaks in A 549 cells at the tested dose with the alkaline comet assay.

Cross-reference to other study
http://www.nanogenotox.eu/

Endpoint study record: Genetic toxicity in vitro_NM 103_COMET_BEAS-2B 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
</tbody>
</table>

Bibliographic source

<table>
<thead>
<tr>
<th>Testing laboratory</th>
<th>NIOM (PL)</th>
<th>Report no.</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Owner company</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Company study no.</td>
<td></td>
<td>Report date</td>
<td></td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Type of genotoxicity

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test guideline

Qualifier no guideline available

Guideline

Deviations

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM 103

Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

Method

Species/strain

Species/strain mammalian cell line, other:

Details on mammalian cell lines (if applicable) human bronchial epithelial BEAS 2B cells

Additional strain characteristics
Metabolic activation

Metabolic activation system

Test concentrations
50/100/256 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time of 3 h and 24 h

Evaluation criteria
Median percentage of DNA in the tail (% Tail intensity) with 200 cells scored

Statistics
ANOVA test with Dunnett’s post-hoc test

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 detailed protocol in annex

Applicant’s summary and conclusion

Interpretation of results
negative

Conclusions
TiO2 NM 103 does not induce DNA strand breaks in the BEAS-2B cells following both 3 h and 24h with the alkaline comet assay

Cross-reference to other study

Endpoint study record: Genetic toxicity in vitro_NM 103_COMET Caco-2 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td>Testing laboratory</td>
<td>NIOM (PL)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D5</td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but not willing to share

Materials and methods

Type of genotoxicity
DNA damage and/or repair

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

Test guideline

Qualifier no guideline available

Guideline

Deviations

Test materials

Test material equivalent to submission substance identity
yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 103

Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

Method

Species/strain

Species/strain mammalian cell line, other:

Details on mammalian cell lines (if applicable)
Undifferentiated human cell line Caco-2

Additional strain characteristics
Metabolic activation

Metabolic activation system

Test concentrations
50/100/256 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time of 3 h and 24 h

Evaluation criteria
percentage of DNA in the tail (% Tail DNA) with 200 cells scored.

Statistics
ANOVA test with Dunnett’s post-hoc test

Attached full study report
Attached document D2_WP4_s SOPs report: ENV/JM/MONO(2015)17/ANN1
Remarks Dispersion protocol
Remarks Data in the report and detailed protocol in annex

Applicant’s summary and conclusion

Interpretation of results
other: Negative at 3 h: no increase in the % Tail DNA at the tested dose Equivocal at 24 h: statistical significant increase in the % Tail DNA at 100 µg/ml

Conclusions
TiO2 NM 103 does not induce DNA breaks in Caco-2 cells at 3 h and induces an equivocal response at 24 h with the alkaline comet assay

Cross-reference to other study

Endpoint study record: Genetic toxicity in vitro_NM 103_COMET NHEK by NANOGENOTOX

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

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

Type of study
single cell gel/comet assay in mammalian cells for detection of DNA damage
Test guideline
Qualifier no guideline available
Guideline
Deviations
Test materials
Test material equivalent to submission substance identity
yes
Reference Material/Nanomaterial and Sample identification number
Identifier Reference Material/Nanomaterial
Identity NM 103
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)
Method
Species/strain
Species/strain mammalian cell line, other: Normal human epidermal keratinocytes (NHEK) from Lonza
Details on mammalian cell lines (if applicable)
Additional strain characteristics
Metabolic activation
Metabolic activation system
Test concentrations
15/33/65 µg/ml
Vehicle
BSA 0.05 % prepared in milliQ water
Details on test system and conditions
single dose with incubation time of 3 h and 24 h
Evaluation criteria
percentage of DNA in the tail (% Tail DNA) with 200 cells scored.

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 detailed protocol in annex
Applicant's summary and conclusion

Interpretation of results
ambiguous increase in the % Tail DNA at both 3h and 24h exposure at one dose only.

Conclusions
TiO2 NM 102 induces an equivocal response in NHEK cells following both 3h and 24 h incubation with the alkaline comet assay.

Cross-reference to other study

Endpoint study record: Genetic toxicity in vitro_NM 103_MLA TK 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>study report</td>
<td>H Norppa</td>
<td></td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bibliographic source</th>
<th>Testing laboratory</th>
<th>Report no.</th>
<th>Owner company</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IPL (F)</td>
<td>DE</td>
<td></td>
</tr>
</tbody>
</table>

Data access
other: Owner: NANOGENOTOX

Data protection claimed
yes, but willing to share

Materials and methods

Type of genotoxicity
gene mutation

Type of study
mammalian cell gene mutation assay
Test guideline

Qualifier according to
Guideline OECD Guideline 476 (In vitro Mammalian Cell Gene Mutation Test) MLA TK

Deviations

Test materials
Test material equivalent to submission substance identity
yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 103

Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Method
Species/strain
Species/strain mammalian cell line, other: L5178Y TK +/- mouse lymphoma cells

Details on mammalian cell lines (if applicable)

Additional strain characteristics

Metabolic activation without

Metabolic activation system

Test concentrations
32/64/128/256/312.5 , 625/1250/2500 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time 24 h

Evaluation criteria
Mutation frequency for Small colonies + Large colonies (x106 cells)

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 detailed protocol in annex

Applicant's summary and conclusion

Interpretation of results
negative
Conclusions

TiO2 NM 103 is not mutagenic in L5178Y TK +/- mouse lymphoma cells at the tested doses with the in vitro mammalian cell gene mutation test.

Cross-reference to other study


**Endpoint study record: Genetic toxicity in vitro_NM 103_MN 16-HBE by NANOGENOTOX**

**Administrative Data**

<table>
<thead>
<tr>
<th>Purpose flag</th>
<th>Study result type</th>
<th>Study period</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ) robust study summary</td>
<td>experimental result</td>
<td>2012</td>
</tr>
</tbody>
</table>

**Data source**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>study report</td>
<td>H norppa</td>
<td>2013</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bibliographic source</th>
<th>Testing laboratory</th>
<th>Report no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IPL (F)</td>
<td>D5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Company</th>
<th>Study no.</th>
<th>Report date</th>
</tr>
</thead>
<tbody>
<tr>
<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**

**Type of genotoxicity**

chromosome aberration

**Type of study**

in vitro mammalian cell micronucleus test

**Test guideline**

<table>
<thead>
<tr>
<th>Qualifier</th>
<th>Guideline</th>
<th>Deviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>according to</td>
<td>other guideline: micronucleus assay (OECD guideline 487)</td>
<td>yes Study without cytochalasin B</td>
</tr>
</tbody>
</table>
Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 103

Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

Method

Species/strain

Species/strain mammalian cell line, other: human bronchial epithelial 16 HBE cells

Details on mammalian cell lines (if applicable)

Additional strain characteristics

Metabolic activation

Metabolic activation system

Test concentrations

64/128/256 µg/ml

Vehicle

BSA 0.05 % prepared in milliQ water

Details on test system and conditions

single dose with incubation time of 41 h

Evaluation criteria

1000 cells scored per culture; 2000 cells scored per condition

Statistics

Chi square

Overall remarks, attachments

Attached full study report

Attached document D2_WP4_s SOPs report: ENV/JM/MONO(2015)17/ANN1

Remarks Dispersion protocol


Remarks Data in the report and detailed protocol in annex

Applicant's summary and conclusion

Interpretation of results

negative

Conclusions

TiO2 NM 103 does not induce aneugenic/clastogenic damage in 16-HBE cells at the tested dose with the
Cytokinesis-block micronucleus assay

Cross-reference to other study

**Endpoint study record: Genetic toxicity in vitro NM 103_MN A549 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**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author</strong></td>
<td>H Norppa</td>
</tr>
<tr>
<td><strong>Year</strong></td>
<td>2013</td>
</tr>
<tr>
<td><strong>Title</strong></td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td><strong>Testing laboratory</strong></td>
<td>RIVM (NL)</td>
</tr>
<tr>
<td><strong>Report no.</strong></td>
<td>D5</td>
</tr>
<tr>
<td><strong>Company study no.</strong></td>
<td>Report date</td>
</tr>
</tbody>
</table>

**Data access**

- other: Owner: NANOGENOTOX

**Data protection claimed**

- yes, but willing to share

**Materials and methods**

**Type of genotoxicity**

- chromosome aberration

**Type of study**

- in vitro mammalian cell micronucleus test

**Test guideline**

- **Qualifier**
  - according to
- **Guideline**
  - other guideline: Cytokinesis-block micronucleus assay (OECD guideline 487)
- **Deviations**
  - yes Cyto B added 6 h after NM

**Test materials**

**Test material equivalent to submission substance identity**

- yes
Reference Material/Nanomaterial and Sample identification number

**Identifier**  Reference Material/Nanomaterial

*Identity*  NM 103

Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

**Method**

**Species/strain**

- Species/strain: mammalian cell line, other: human alveolar epithelial A549 cells

Details on mammalian cell lines (if applicable)

Additional strain characteristics

Metabolic activation

Metabolic activation system

**Test concentrations**

2/4/8/16/32/64/128/256/512 µg/ml

**Vehicle**

BSA 0.05 % prepared in milliQ water

**Evaluation criteria**

1000 cells scored per culture; 2000 cells scored per condition

**Statistics**

Chi square

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 detailed protocol in annex

Applicant's summary and conclusion

**Interpretation of results**

negative

**Conclusions**

TiO2 NM 103 does not induce aneugenic/clastogenic damage in A 549 cells at the tested dose with the Cytokinesis-block micronucleus assay

Cross-reference to other study

Endpoint study record: Genetic toxicity in vitro_NM 103_MN Caco-2 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td>Testing laboratory</td>
<td>Anses (F)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D5</td>
</tr>
<tr>
<td>Owner company</td>
<td></td>
</tr>
<tr>
<td>Company study no.</td>
<td></td>
</tr>
<tr>
<td>Report date</td>
<td></td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Type of genotoxicity
chromosome aberration

Type of study
in vitro mammalian cell micronucleus test

Test guideline

Qualifier according to
Guideline other guideline: Cytokinesis-block micronucleus assay (OECD guideline 487)
Deviations yes Cyto B added 24 h after NM

Test materials

Test material equivalent to submission substance identity
yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 103
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Method
Species/strain

- mammalian cell line, other: Undifferentiated human cell line Caco-2

Details on mammalian cell lines (if applicable)

Additional strain characteristics

Metabolic activation

Metabolic activation system

Test concentrations
9.5/28/85/128/256 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time of 52 h

Evaluation criteria
1000 cells scored per culture; 2000 cells scored per condition

Statistics
Chi square

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 detailed protocol in annex

Applicant's summary and conclusion

Interpretation of results
negative

Conclusions
TiO2 NM 103 does not induce aneugenic/clastogenic damage in Caco-2 cells at the tested dose with the Cytokinesis-block micronucleus assay.

Cross-reference to other study
Endpoint study record: Genetic toxicity in vitro_NM 103_MN BEAS-2B 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>FIOH (FL)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D5</td>
</tr>
<tr>
<td>Owner company</td>
<td></td>
</tr>
<tr>
<td>Company study no.</td>
<td></td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Type of genotoxicity

chromosome aberration

Type of study

in vitro mammalian cell micronucleus test

Test guideline

Qualifier according to

Guideline other guideline: Cytokinesis-block micronucleus assay (OECD guideline 487)

Deviations yes Cyto B added 6 h after NM

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM 103
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Method
Species/strain
Species/strain mammalian cell line, other: human bronchial epithelial BEAS 2B cells
Details on mammalian cell lines (if applicable)
Additional strain characteristics
Metabolic activation
Metabolic activation system
Test concentrations
32/64/128/256 µg/ml
Vehicle
BSA 0.05 % prepared in milliQ water
Details on test system and conditions
single dose with incubation time of 48 h
Evaluation criteria
1000 cells scored per culture; 2000 cells scored per condition
Statistics
Chi square

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 detailed protocol in annex

Applicant's summary and conclusion
Interpretation of results
negative

Conclusions
TiO2 NM 103 does not induce aneugenic/clastogenic damage in Beas-2B cells at the tested dose with the Cytokinesis-block micronucleus assay.

Cross-reference to other study
**Endpoint study record: Genetic toxicity in vitro_NM 103_MN Lymphocytes 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**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>INSA (PT)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D5</td>
</tr>
<tr>
<td>Owner company</td>
<td></td>
</tr>
<tr>
<td>Company study no.</td>
<td></td>
</tr>
<tr>
<td>Report date</td>
<td></td>
</tr>
</tbody>
</table>

**Data access**

other: Owner: NANOGENOTOX

**Data protection claimed**

yes, but willing to share

**Materials and methods**

**Type of genotoxicity**

chromosome aberration

**Type of study**

in vitro mammalian cell micronucleus test

**Test guideline**

- **Qualifier** according to
- **Guideline** other guideline: Cytokinesis-block micronucleus assay (OECD guideline 487)
- **Deviations** yes Cyto B added 6 h after NM

**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>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>NM 103</td>
</tr>
</tbody>
</table>
Details on test material

Commercial name: UV TITAN M262 (Sachtleben)

Method

Species/strain

Species/strain: primary culture, other: human primary peripheral blood lymphocytes

Additional strain characteristics

Metabolic activation

Metabolic activation system

Test concentrations

5/15/45/125/250 µg/ml

Vehicle

BSA 0.05 % prepared in milliQ water

Details on test system and conditions

single dose with incubation time of 30 h with Cyto B added after 6 h exposure with NM

Evaluation criteria

1000 cells scored per culture; 2000 cells scored per condition

Statistics

Chi square

Applicant's summary and conclusion

Interpretation of results

positive Statistical significant increase in the frequency of binucleated cells with micronuclei measured at two doses (5 and 45 µg/ml)

Conclusions

TiO2 NM 103 induces aneugenic/clastogenic damage at 5 and 45 µg/ml in the human blood lymphocytes with the Cytokinesis-block micronucleus assay.

Overall remarks, attachments

Attached full study report


Remarks: Dispersion protocol


Remarks: Data in the report and detailed protocol in annex

Cross-reference to other study

**Endpoint study record: Genetic toxicity in vitro_NM 103_MN NHEK 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**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>H Norppa</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 5: In vitro testing strategy for nanomaterials including database</td>
</tr>
<tr>
<td>Testing laboratory</td>
<td>IMB-BAS (BG)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D5</td>
</tr>
</tbody>
</table>

**Data access**

- other: Owner: NANOGENOTOX

**Data protection claimed**

- yes, but willing to share

**Materials and methods**

**Type of genotoxicity**

- chromosome aberration

**Type of study**

- in vitro mammalian cell micronucleus test

**Test guideline**

- **Qualifier**: according to
- **Guideline**: other guideline: Cytokinesis-block micronucleus assay (OECD guideline 487)
- **Deviations**: yes Cyto B added 6 h after NM

**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>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>NM 102</td>
</tr>
</tbody>
</table>
Details on test material
Commercial name: UV TITAN M262 (Sachtleben)

Method
Species/strain
Species/strain mammalian cell line, other: Normal human epidermal keratinocytes (NHEK) from Lonza

Details on mammalian cell lines (if applicable)
Additional strain characteristics

Metabolic activation

Metabolic activation system

Test concentrations
7.5/37.5/75 µg/ml

Vehicle
BSA 0.05 % prepared in milliQ water

Details on test system and conditions
single dose with incubation time of 54 h

Evaluation criteria
1000 cells scored per culture; 2000 cells scored per condition

Statistics
Chi square

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 detailed protocol in annex

Applicant's summary and conclusion

Interpretation of results
positive Dose-dependant increase in the frequency of binucleated cells with micronuclei

Conclusions
TiO2 NM 103 induces aneugenic/clastogenic damage in NHEK cells with the Cytokinesis-block micronucleus assay.

Cross-reference to other study
7.6.2 Genetic toxicity in vivo

*Endpoint study record: Genetic toxicity in vivo_NM 103_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**

<table>
<thead>
<tr>
<th>Reference type</th>
<th>Author</th>
<th>Year</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>study report</td>
<td>V Fessard</td>
<td>2013</td>
<td>Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bibilographic source</th>
<th>Testing laboratory</th>
<th>Report no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NRCWE (DK)</td>
<td>D6</td>
</tr>
</tbody>
</table>

**Data access**

- Owner: NANOGENOTOX
- yes, but willing to share

**Cross-reference to same study**

NM103_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 guideline**

- **Qualifier**: no guideline available
- **Guideline**
- **Deviations**
Test materials

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

**Identifier**  
Reference Material/Nanomaterial

**Identity**  
NM 103

**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: 1st at 0, 2nd at 24h and the 3rd at 45 h Sampling 3 h after the last administration

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

**Basis**  
nominal conc.

**No. of animals per sex per dose**  
5

**Control animals**  
yes

**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


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-103 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 103_MN 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>V Fessard</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis</td>
</tr>
<tr>
<td>Bibliographic source</td>
<td>NRCWE (DK)</td>
</tr>
<tr>
<td>Report no.</td>
<td>D6</td>
</tr>
<tr>
<td>Company study no.</td>
<td>Report date</td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share
Cross-reference to same study
NM103_COMET Instillation_NRCWE

Materials and methods

Type of genotoxicity
cromosome aberration

Type of study
micronucleus assay

Test guideline

Qualifier according to
Guideline OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)

Deviations

Test materials

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 103

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: 1st at 0, 2nd at 24h and the 3rd at 45 h Sampling: 3 h after the last administration

Doses / concentrations
1.15, 2.3, 4.6 mg/kg

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

Statistics
Chi square test

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
Interpretation of results
negative

Conclusions
TiO2 NM-103 is not genotoxic at the tested doses following a short-term exposure via intratracheal instillation with the micronucleus assay in bone marrow.

Cross-reference to other study

Endpoint study record: Genetic toxicity in vivo NM 103 COMET Gavage 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>V Fessard</td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis</td>
</tr>
</tbody>
</table>

Bibliographic source

<table>
<thead>
<tr>
<th>Testing laboratory</th>
<th>IMB-BAS (BG)</th>
<th>Report no.</th>
<th>D6</th>
</tr>
</thead>
</table>

| Owner company      |              |

| Company study no.  |              |

Data access

other: owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Cross-reference to same study

NM103_MN Bone marrow Gavage

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 guideline

Qualifier: no guideline available

Guideline: Deviations

Test materials

Reference Material/Nanomaterial and Sample identification number

Identifier: Reference Material/Nanomaterial

Identity: NM 103

Test animals

Species: rat

Strain: Sprague-Dawley
Sex
male

Administration / exposure

Route of administration
oral: gavage

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: 1st at 0, 2nd at 24h and the 3rd at 45 h
Sampling: 3 h after the last administration

Doses / concentrations
6, 12 and 24 mg/kg bw/d TiO2 for colon and intestine; 6.5, 13, 26 mg/kg bw/d TiO2 for all other organs

No. of animals per sex per dose
5

Control animals
yes

Positive control(s)
Methyl MethaneSulfonate 280 mg/kg bw/d

Examinations

Tissues and cell types examined
intestine, colon, blood, bone marrow, spleen, liver, kidney

Evaluation criteria
Median % Tail intensity from >100 cells per organ

Statistics
Mann–Whitney U test followed by Jonckheere-Terpstra trend test

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 porocol in annex

Applicant's summary and conclusion

Interpretation of results
other: Positive in intestine : Dose-dependant increase in the % Tail intensity with the highest dose being significant Equivocal in spleen: Increase in the % Tail intensity at one dose only Negative in colon, blood, bone marrow, spleen, liver, kidney
Conclusions
TiO2 NM-103 is genotoxic in the intestine and induces equivocal response in the spleen of rats following a short-term exposure via gavage with the alkaline comet assay

Cross-reference to other study

Endpoint study record: Genetic toxicity in vivo_NM 103_MN Bone marrow Gavage by NANOGENOTOX

Administrative Data

<table>
<thead>
<tr>
<th>Purpose flag</th>
<th>( ) robust study summary ( ) used for classification ( ) used for MSDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study result type</td>
<td>experimental result                                    Study period</td>
</tr>
</tbody>
</table>

Data source

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>2013</td>
</tr>
<tr>
<td>Title</td>
<td>Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis</td>
</tr>
</tbody>
</table>

Bibliographic source

<table>
<thead>
<tr>
<th>Testing laboratory</th>
<th>IMB-BAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Report no.</td>
<td>D6</td>
</tr>
<tr>
<td>Owner company</td>
<td></td>
</tr>
</tbody>
</table>

Data access

other: Owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Cross-reference to same study

NM103_COMET Gavage_IMB

Materials and methods

Type of genotoxicity
chromosome aberration

Type of study
micronucleus assay
Test guideline
Qualifier according to
Guideline OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)

Deviations

Test materials
Reference Material/Nanomaterial and Sample identification number
Identifier Reference Material/Nanomaterial
Identity NM 103

Test animals
Species
rat

Strain
Wistar

Sex
male

Administration / exposure
Route of administration
oral: gavage

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: 1st at 0, 2nd at 24h and the 3rd at 45 h Sampling 3 h after the last administration

Doses / concentrations
24 mg/kg bw/d

Basis nominal conc.

No. of animals per sex per dose
5

Positive control(s)
Methyl MethaneSulfonate 280 mg/kg bw/d

Examinations
Tissues and cell types examined
Bone marrow

Evaluation criteria
2000 immature erythrocytes per rat

Statistics
Chi square test
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

Interpretation of results

negative

Conclusions

TiO2 NM-103 is not genotoxic in rats at the tested dose following a short-term exposure via gavage with the micronucleus assay in bone marrow

Cross-reference to other study


**Endpoint study record: Genetic toxicity in vivo NM 103 MN Bone marrow IV 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

<table>
<thead>
<tr>
<th>Reference type</th>
<th>study report</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>V Fessard</td>
<td>2013</td>
</tr>
</tbody>
</table>

| Title | Deliverable 6: Characterisation of manufactured nanomaterials for their clastogenic/aneugenic effects or DNA damage potentials and correlation analysis |

<table>
<thead>
<tr>
<th>Bibliographic source</th>
<th></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Testing laboratory</th>
<th>Report no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIVM (NL)</td>
<td>D6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Owner company</th>
<th>Company study no.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Data access</th>
<th>other: Owner: NANOGENOTOX</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Data protection claimed</th>
<th>yes, but willing to share</th>
</tr>
</thead>
</table>
Materials and methods

Type of genotoxicity
chromosome aberration

Type of study
micronucleus assay

Test guideline
 Qualifier according to
  Guideline OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)

Deviations

Test materials

Test material equivalent to submission substance identity
yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial
Identity NM 103

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
intravenous

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

Duration of treatment / exposure
1 single administration or repeated administration (once a day for 5 consecutive days)

Doses / concentrations
8.7 – 9.7 mg/kg body weight (b.w.) for male animals, and 12.4 – 13.7 mg/kg b.w. for female
Basis nominal conc.

No. of animals per sex per dose
2-6

Positive control(s)
Methyl Methane Sulfonate 25 mg/kg

Examinations
Tissues and cell types examined
Bone marrow

Evaluation criteria
2000 immature erythrocytes per rat

Statistics
Chi square test

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

Interpretation of results
Negative

Conclusions
TiO2 NM-103 is not genotoxic at the tested doses in rats exposed via intravenous route, irrespective of the gender and the duration of exposure with the micronucleus assay in bone marrow.

Cross-reference to other study

7.6.3 Photogenotoxicity

Summary of the HH literature data status 03rd April 2014: Summary of studies on Photogenotoxicity

<table>
<thead>
<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>
</tr>
</thead>
<tbody>
<tr>
<td>SCCNFP 2000</td>
<td>UV TITAN M 262, rutile, 20 nm, coated (Kemira)</td>
<td>S. typhimurium and E. coli</td>
<td>Ames test</td>
<td>?</td>
<td>negative</td>
</tr>
</tbody>
</table>
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 103 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

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).

199
Applicant's summary and conclusion

Cross-reference to other study

8. ANALYTICAL METHODS

9. RESIDUES IN FOOD AND FEEDINGSTUFFS

10. EFFECTIVENESS AGAINST TARGET ORGANISMS

11. GUIDANCE ON SAFE USE