Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

17-Sep-2015

English - Or. English

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

DOSSIER ON TITANIUM DIOXIDE - PART 2 - NM 100

Series on the Safety of Manufactured Nanomaterials
No. 54

This document is only available in PDF format.

JT03381841

ENV/JM/MONO(2015)17/PART2
Unclassified

English - Or. English

OECD Environment, Health and Safety Publications

Series on the Safety of Manufactured Nanomaterials

No. 54

DOSSIER ON TITANIUM DIOXIDE - PART 2 - NM 100

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2015

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)*

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.

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PREAMBLE

In November 2007, OECD's Working Party on Manufactured Nanomaterials (WPMN) launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The outcomes of the Testing Programme were intended to assess the applicability of the existing ***test guidelines***[1] 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 testing[2]. This list comprised: i) fullerenes (C60); ii) single-walled carbon nanotubes (SWCNTs); iii) multi-walled carbon nanotubes (MWCNTs); iv) silver nanoparticles; v) titanium dioxide; vi) cerium oxide; vii) zinc oxide; viii) silicon dioxide; ix) dendrimers; x) nanoclays; and xi) gold nanoparticles. One fundamental criterion for selecting these materials was that they should be either in commercial use at the time or expected to be in the near future. At the same time, other considerations were also given attention, such as the production volume of the materials, the likely availability of such materials for testing and the existing information that would readily be available on the materials.

It was also agreed that 59 endpoints would be addressed[3] 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 sponsors[5] in the testing of the materials while ensuring that the information collected was reliable, accurate, consistent and therefore also comparable. The Guidance Manual addressed a whole range of issues including the organisation of the work.

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

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

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

The work on OECD's Testing Programme was completed by the end of 2013. In June 2014 the WPMN agreed that for each nanomaterial the dataset would be published in IUCLID printed 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.

FOREWORD

As part of its Programme on the Safety of Manufactured Nanomaterials, OECD launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The Testing Programme mainly aimed to assess the applicability of the existing test guidelines to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions.

This document presents the Dossier of the Titanium Dioxide (TiO_2) manufactured nanomaterials which was prepared under the leadership of France and Germany. TiO2 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[8] style format and includes the protocols and methods used (see Preamble).They are resulting from scientific literature and testing following harmonised guideline or protocols (like OECD Guidelines for the Testing of Chemicals)[9], or not

France and Germany led the Testing Programme on nano-TiO_2. This included the determination of data from the tests already completed using nano-TiO_2, a number of new tests from dedicated research project, as well as coordinating inputs provided and tests performed by other participating countries and stakeholder from Austria, Canada, Denmark, Spain, Japan, Korea, United Kingdom, United States, European Union, and the Business and Industry Advisory Committee to the OECD (BIAC).

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

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

At the same time, it was recognised that the nano-TiO_2 placed on the market presents high variability in its composition. With this in mind, additional materials were selected for performing a selected number of endpoints that could allow some comparability. As a consequence this allowed testing a broad range of material's characteristics and covering a broader range of exposure scenarios to human and the environment. These materials were:

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

[9] http://www.oecd.org/env/testguidelines

- 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 litterature. 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 metrelogie et d"Essais, Laboratoire National de metrelogie et d"Essais

NIOM, The Nofer Institute of Occupational Medicine (Poland), The Laboratory of Molecular Toxicology

NRCWE, National Research Centre for the Working Environment (Denmark), Nanotoxicology and Occupational Hygiene Group

RIVM, National Institute for Public Health and Environment (The Netherlands), The Laboratory for Health Protection Research

UAB, Universitat Autonoma de Barcelona (Spain), The Group of Mutagenesis

Germany:

Institute of Energy and Environmental Technology (IUTA)., Air Quality & Sustainable Nanotechnology

Fraunhofer Institute of Toxicology & Experimental Medicine, Inhalation Toxicology & Chemical Risk Assessment

Fraunhofer Institute for Molecular Biology and Applied Ecology

RWTH Aachen, Institute of Ecochemistry,Ecology, and Ecotoxicology

University Frankfurt Main, Institute for Ecology, Evolution and Diversity

Technical University Dresden, Institute of process engineering and environmental technology

Hamburg University of Applied Sciences

Federal Institute for Materials Research and Testing, Materials and Air Pollutants

Japan:

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

Korea:

Dongduk Women's University, College of Pharmacy

Hanyang University, Laboratory of Nanoscale Characterisation & Environmental Chemistry

Korea Research Institute of Standards and Science, Korea Research Institute of Standards and Science Division of Industrial Metrology

Seoul National University, School of Chemical & Biological Engineering

Kyung Hee University, Department of Applied Chemistry

Korea University, School of Life Science & Biotechnology

Spain:

INIA, Departamento de Medio Ambiente

USA:

NIST, Nanoparticle Measurements & Standards

EPA, National Health and Environmental Effects Research

EPA, Ecology Division

FDA, National Center for Toxicological Research

Finally, we would also like to acknowledge the effort done by the EC/Joint Research Centre in providing the materials, homogenised, sub-sampled and kept them under inert atmosphere before they delivered them to participating laboratories; as well as TDMA in providing some of the materials to JRC.

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Endpoint study record: Particle size, size distribution by University of Graz

Administrative Data

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

Data source

Data access
other: performed and provided by University of Graz, Austria

Cross-reference to same study

[1] Roblegg E., Fröhlich E., Samberger C., Zaversky M., Teubl B., Zimmer A., Evaluation of a physiological in-vitro system to study the transport of nanoparticles through the buccal mucosa, Nanotoxicology. 2011 May 18. [Epub ahead of print] PMID: 21591874
[2] Teubl B., Meindl C., Eitzelmayer A., Zimmer A., Fröhlich E. and Roblegg E., In-vitro Permeability Studies of neutrally charged Polystyrene Particles through the Buccal Mucosa, submitted 2012
[3] Teubl B., Leitinger G., Fröhlich E., Schneider M., Tockner M., Zimmer A., Roblegg E., Buccal Mucosa as a Route for TiO2 Uptake, in preparation

Materials and methods

Methods
TEM

Results and discussions

Remarks on results including tables and figures
Characterization via TEM: Mean particles size (MQ water): 148.25 and 33.5 (two size distributions)

Overall remarks, attachments
Attached background material

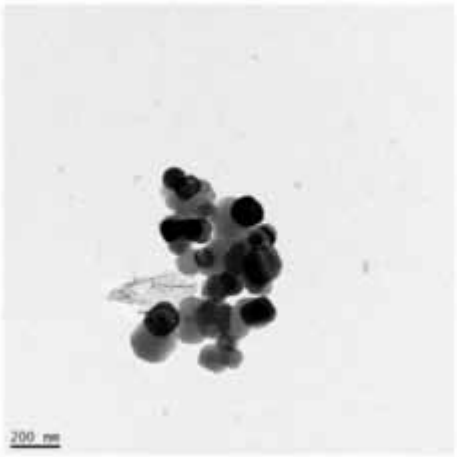


Figure 1 TEM image of NM100 dispersed in MQ water

Endpoint study record: TEM by University of Graz (2)

Administrative Data

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

Data source

Data access

other: performed and provided by University of Graz

Materials and methods

Methods

TEM

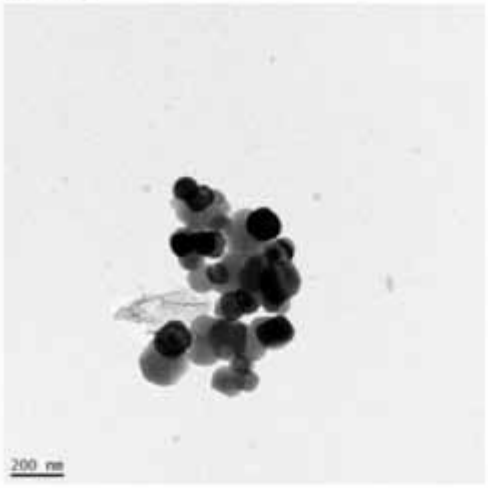
Overall remarks, attachments

Attached background material

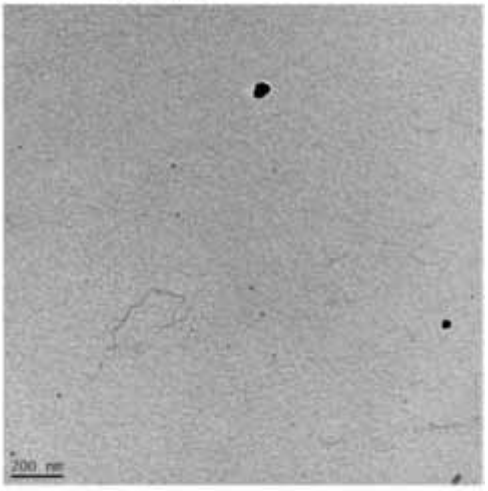
Sponsorship Program: Titanium Dioxide Report

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

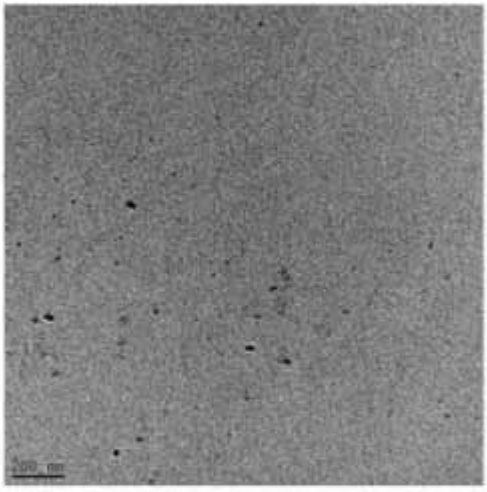
NM 100



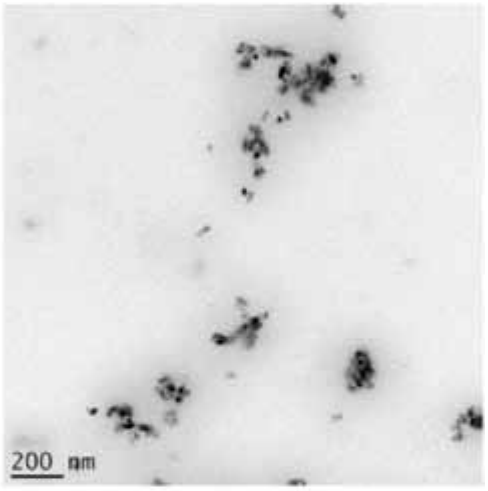
NM 101



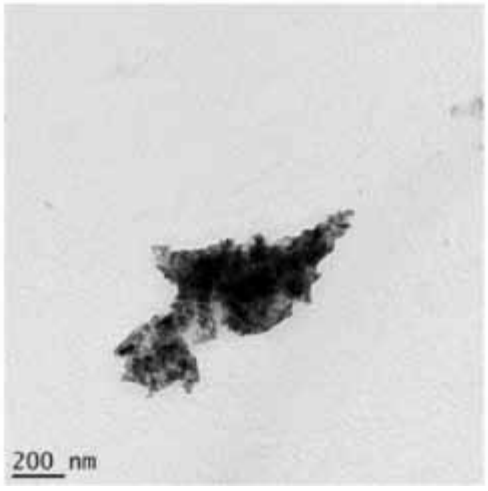
NM 102



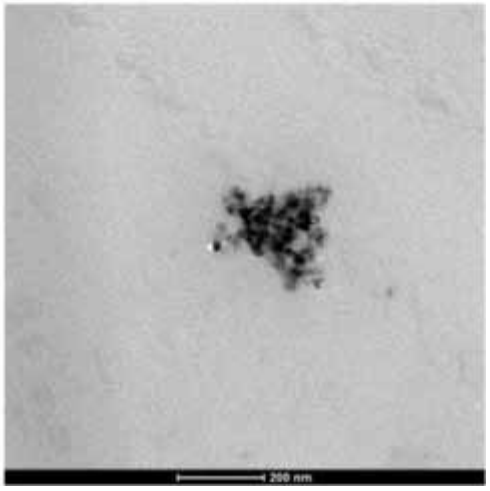
NM 103



NM 104



NM 105



Endpoint study record: Particle size, size distribution by University of Applied Science Hamburg (HAW Hamburg)

Administrative Data

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

Data source

Reference

Reference type publication

Author Judith Angelstorf **Year** 2013
Title PhD thesis, in preparation
Bibliographic source
Testing laboratory HAW Hamburg, TUHH Hamburg
Report no.
Owner company
Company study no.

Report date

Data access

other: performed and provided by HAW Hamburg

Cross-reference to same study

Angelstorf, J.S.1, Ahlf, W. 2; and Heise, S. 1 unpublished data.
1) University of applied scienes Hamburg, Lohbrügger Kirchstraße 65, 21033 Hamburg
2) Technical University of Hamburg-Harburg (TUHH)
Contact: Judith.angelstorf@haw-hamburg

Materials and methods

Methods

SEM

Results and discussions

Mean diameter

>= 90 — <= 230 nm

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

Reference type	study report		
Author	Keld Alstrup Jensen	**Year**	2012
Title	D4.2: Transmission electron microscopic characterisation of NANOGENOTOX nanomaterials. Key intrinsic physicochemical characteristics of NANOGENOTOX nanomaterials		
Bibliographic source	NANOGENOTOX Delivrable no. 5 Final Report		
Testing laboratory	CODA-CERVA (B), INRS (F), IMC-BAS (BG)	**Report no.**	D4.2
Owner company			
Company study no.		**Report date**	2012-05-01

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 perfocmance, 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 maxiumum projected lenght 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.
o 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)
o Descriptive statistics and histograms were calculated in Sigmaplot (Systat, Cosinus computing, Drunen, The Netherlands).
o 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.
o 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.
o The normality of the distributions and the homogeneity of variances were met for the mean values of the median mean diameter.
o 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
o the SAS statistical software (SAS Institute Inc., Cary, NC, USA).
o 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 Vibracell™ 75041 ultrasonifier (750 W, 20kHZ, Fisher Bioblock Scientific, Aalst, Belgium) equipped with a 13 mm horn (CV33) at 40% amplitude. This setup resulted in an average horn power of about 26 W and a sample specific energy of 2530 ± 20 MJ/m³. During sonication the samples were cooled in icy water with ice to prevent excessive heating. After sonication, the samples were diluted to a concentration of 0.512 mg/ml.
Details of used procedure can be found in the nanogenotox dispersion protocol file.

2. Grid adjustment
The charge of grid whas adjustes 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 pioloform- and carbon-coated grids that tend to have a negative or neutral charge. (authors handexpirience 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 measurments the suspended NM was brought on pioloform- and carbon- coated, 400 mesh copper grids (Agar Scientific, Essex, England) that were pretreated with 1% Alcian blue (Fluka, Buchs, Schwitzerland).
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 detailes 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

Detals for calibration in Semi-automaitc 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 Measurments, 1Edn. Pijnacker: Springer 2009) that large systematic size deviations can be avoided if the particle area is at least hundred pixels.)
• The field of view limits the upper size detection limit to 245 nm, one tenth of the image size as recommended in ISO 13322-1 (part 1, 2004)

Reproducibility

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM-100

State of test material

other: fluffy powder

Results and discussions

NM100 contains the largest primary particles and the material consist of mainly aggregated euhedral ca. 50 to 200 nm-size anatase crystals (Figure 8). The average primary particle size of NM100 is on the order of 50 to 150 nm, depending on the laboratory (Table 3).

Remarks on results including tables and figures

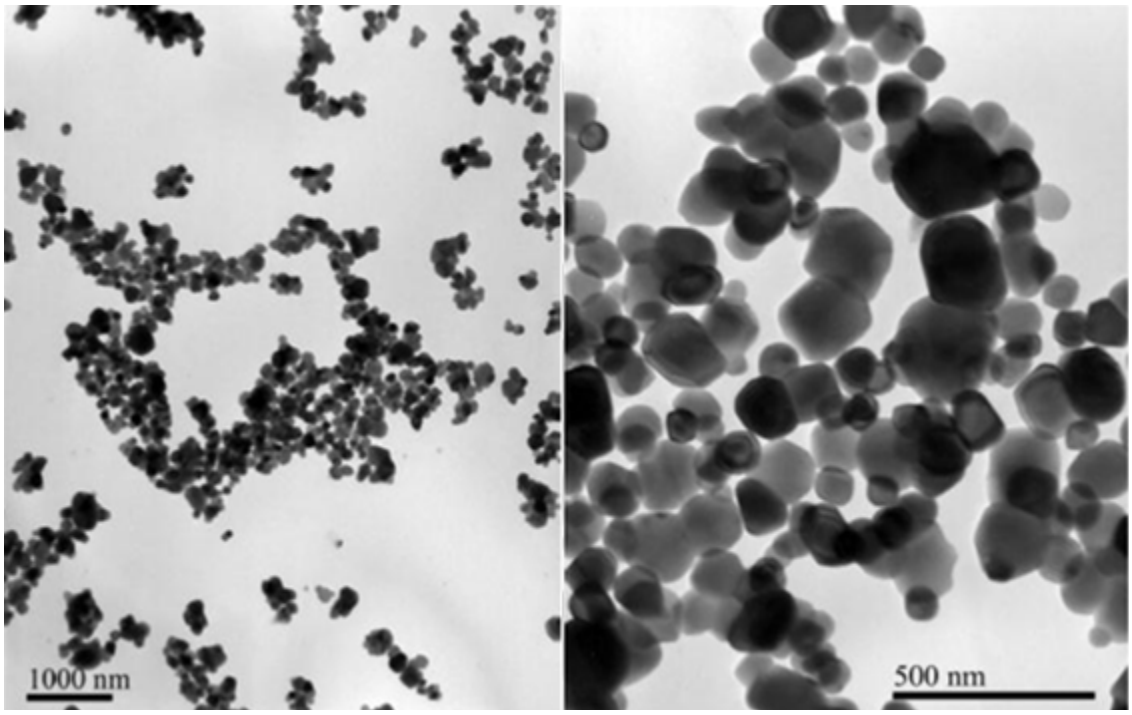


Figure 1 NM100: A) Representative TEM-micrograph showing the range in agglomerate and aggregate sizes and typical euhedral morphology of the individual crystallites in the sample. B) selected TEM-micrograph taken at higher resolution illustrating the aggregates are mainly aggregates sintered at crystal facets.

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

Sample	ECD (nm) ± SD (N); CODA-CERVA	ECD (nm) ± SD (N); INRS	Diameter (nm); IMC-BAS
NM-100	50-90*		116.9 ± 36.8**

**** Manual measurement***

***** Manual measurement using ImageJ software.***

Overall remarks, attachments

Attached full study report

Draft_D4.2_TEM characterisation.pdf / 2.31 MB (application/pdf):
ENV/JM/MONO(2015)17/ANN4

Applicant's summary and conclusion

Conclusions

very good correspondence between AFM and TEM values

Cross-reference to other study

http://www.nanogenotox.eu/: **ENV/JM/MONO(2015)17/ANN22**

4.6 Vapour pressure

4.7 Partition coefficient

4.8 Water solubility

4.9 Solubility in organic solvents / fat solubility

4.10 Surface tension

4.11 Flash point

4.12 Auto flammability

4.13 Flammability

4.14 Explosiveness

4.15 Oxidising properties

4.16 Oxidation reduction potential

4.17 Stability in organic solvents and identity of relevant degradation products

4.18 Storage stability and reactivity towards container material

4.19 Stability: thermal, sunlight, metals

4.20 pH

4.21 Dissociation constant

4.22 Viscosity

4.23 Additional physico-chemical information

Endpoint study record: composition by TGA by NANOGENOTOX

Administrative Data

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

Study result type experimental result **Study period** 2013

Data source

Reference

Reference type	study report		
Author	KA Jensen	**Year**	
Title	Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials		
Bibliographic source			
Testing laboratory	NRCWE (DK)	**Report no.**	D4.3
Owner company			
Company study no.		**Report date**	

Data access

other: owner: NANOGENOTOX

Materials and methods

Endpoint investigated

other: mass lost by TGA

Details on methods and data evaluation

In a thermograviometric measurement a sample is heated in a gas (usually air, O2 or N2) and theweight of the sample is measured as a function of the temperature. The decomposition temperatureand loss of mass may give information about the sample, e.g. water adsorbed to the surface ofparticles will evaporate around 100 °C, whereas most other associated or technically added organiccoatings will evaporate or combust at higher temperature. A decomposition in several steps will indicate a non-homogeneous samplecontaining several different types combustable compounds, which could in fact all be structurallydifferent carbon nanotubes.Instruments:For the thermogravimetric analysis (TGA) NRCWE used a Mettler Toledo TGA/SDTA 851e and anoxygen atmosphere. The heating rate was 10 K/min and the same temperature rangefrom 25 °C to 1000 °C. The sample holders used for the TGA measurements were made of aluminaand 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-100

Any other information on materials and methods incl. tables

The SOP used for TGA anlysis:Thermogravimetric Analysis (TGA)Renie Birkedal (NRCWE) based on NIST Recommended Practice Guide, Special Publication960-19General descriptionTGA is short for thermograviometric analysis. The principle is measuring sample weight as a functionof 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 aboutorganic content is wanted heating in O2 or air is recommended, as this will insure combustion of

allorganic material. In order to make sure e.g. all organic material is decomposed, it is recommended torun to 1000 C.Materials and Chemicals:Powder (may be conditioned in a specific atmosphere and humidity conditions)Laboratory weigh (scale)Apparatus for thermogravimetric analysisProcedureSample preparation:♣ Weigh container.♣ Fill container with material. Do not stamp it, as this may affect theevaporation/decomposition temperature.♣ Weigh container and material.For inorganic powder materials a minimum of 10 mg should be used – if possible more. Thesesamples 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 bedifferent. At the same time these compounds often have a low density, and it is therefore difficult tomeasure a representative fraction in one or two measurements. The solution is many measurementsand comparison of the data.Selection of heating rate.For inorganic materials only a minor fraction is expected to decompose, and a heating rate of 10C/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 theweight losses are not well defined a slower heating rate can be chosen.The NIST Recommended Practice Guide, Special Publication 960-19, Measurement Issues in SingleWall Carbon Nanotubes, recommendes a heating rate of 5 C/min. This is chosen as a compromisebetween time and avoiding too much spontaneous combustion. For some carbon nanotubes 5 C/minis not slow enough to avoid spontaneous combustion. There is no spontaneous combustion with aheating rate of 2.5 C/min. The measurement time is very long, app 7 hours per measurement, butthis is still recommended. In order to minimize measuring time it may be an option only to heat to900 C or even lower.Data treatment:Compare TGA curve and curve for first derivative to find steps of weight loss. It is recommended toobtain several measurements to calculate the mean and standard deviation of the weight loss andthe evaporation/decomposition temperatures. (the last is most easily found from the curve of thefirst 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 NM100, the weight change is due to buoyancy (Figure 4-1)

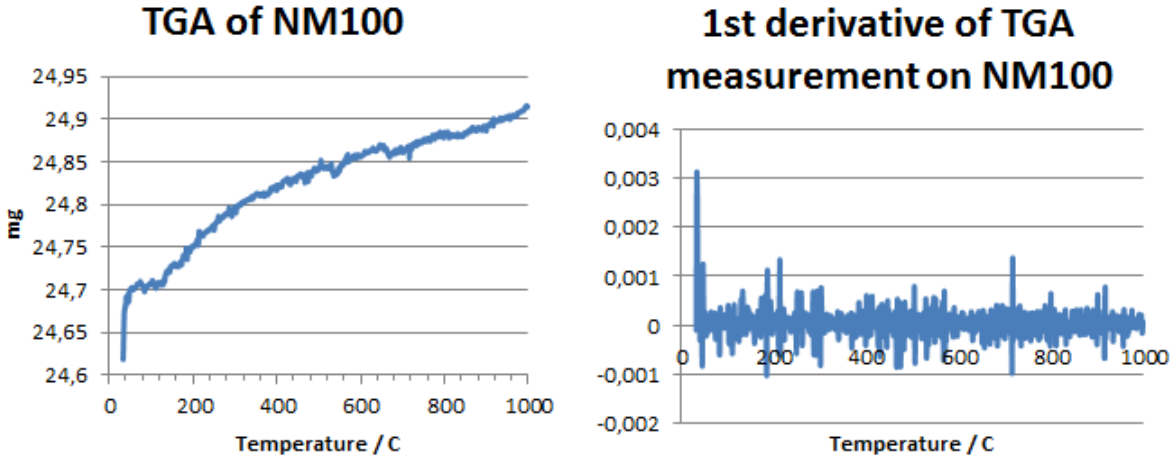


Figure 4-2. Results from TGA measurement on NM100..

Overall remarks, attachments

Attached full study report

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

Remarks Dispersion protocol

Attached document D4.3_MinChemComposition/ D2_WP4_ SOPs report: **ENV/JM/MONO(2015)17/ANN5**

Remarks Data in the report and detailes porocol in annex

Applicant's summary and conclusion

Conclusions

No mass loss observed. The weight change is due to buoyancy

Cross-reference to other study

http://www.nanogenotox.eu/ : **ENV/JM/MONO(2015)17/ANN22**

Endpoint study record: composition by EDS by NANOGENOTOX

Administrative Data

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

Study result type experimental result

Data source

Reference

Reference type	study report		
Author	KA Jensen	**Year**	2013
Title	Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials		
Bibliographic source			
Testing laboratory	IMC-BAS (BG)	**Report no.**	D4.3
Owner company			
Company study no.		**Report date**	

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 toolin electron microscopes.The analysis is based on the fact that when hitting a material with charged particles, such as anelectron beam, some of the electrons of the atoms in the matter under the beam will first beenergized to higher orbital positions and then drop down to their appropriate energy level againduring which X-rays are emitted. The emitted X-rays are characteristic for each element and havespecific energetic wavelengths and energy patterns. Therefore an elemental composition can bequantified 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 bequantified depending detectors and instrumental configuration. Oxygen is normally not analysed bySEM EDS, but may be calculated by difference or by converting all elements to oxides. Whencalculated 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 eitherstandardless/internal instrument standard values or calibrated concentration-intensity curves using arange 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-buildstandard references, such results are relatively reliable for major elements if the materials havesufficiently high thickness and low roughness.Samples were prepared by pelletizing a known amount of powder. The results are given in wt.% andparts per million (ppm) depending on the absolute concentrations in the sample materials.

Test materials

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM 100

Results and discussions

Results

NM100 contains 2100 to 4900 ppm Fe, as well as Si, K and P as well as Al and trace of Cr making 0.86 wt% of the sample.

Remarks on results including tables and figures

Table 5-2 Elemental concentrations by EDS measurements on TiO_2 performed at IMC-BAS.

Sample	Al*	Si*	P*	S*	K*	Ti (wt%)	Cr*	Fe*	O wt%
NM100	900	2800	2100		2500	58.57	300	4900	40.08

Overall remarks, attachments

Attached full study report

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

Remarks Dispersion protocol

Attached document D4.3_MinChemComposition: **ENV/JM/MONO(2015)17/ANN5**

Remarks Data in the report and detailes porocol 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

http://www.nanogenotox.eu/: **ENV/JM/MONO(2015)17/ANN22**

Endpoint study record: composition by ICP_OES by NAOGENOTOX

Administrative Data

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

Study result type experimental result

Data source

Reference

Reference type	study report		
Author	KA Jensen	**Year**	2013
Title	Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials		
Bibliographic source			
Testing laboratory	CODA-CERVA (B)	**Report no.**	
Owner company			
Company study no.		**Report date**	

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 afast estimate of the concentration of non-calibrated compounds in samples. The samples werescreened 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 (SCPSCIENCE) and 2 ml of concentrated HF was added. The mixture was heated overnightat 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-100

Results and discussions

Results

Tables 5-8 and 5-9 show the elemental concentration ranges found after screening the TiO_2 samples by ICP-OES. No elements in NM102 and NM105 were found to be present in concentrations higher than 0.1 wt%. Only K was found in concentrations between 0.1 and 1 wt% in NM100. Na, P, Ca, and Zr were found in trace amounts. Notably trace amounts of Zr were found in all samples, but NM105. The most anundant impurities (> 1 wt%) were found to be Al in NM103 and NM104. Na and K (both 0.1–1 wt%) were the most abundant impurities in NM102 and NM100, respectively.

Table 5-3. Graphical summary table with the impurity ranges found in titanium dioxide.

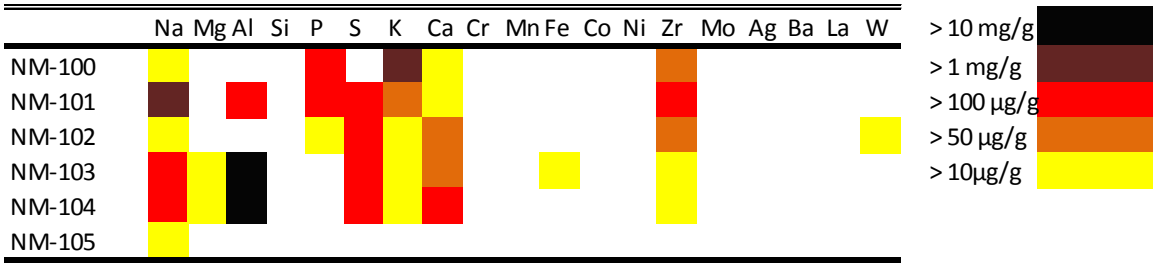


Table 5-4. Overview of impurities detected in titanium dioxide NM.

Nanomaterial	Vial ID n°	Impurities > 0.01%	Impurities 0.005 – 0.01%	Impurities 0.001 – 0.005%
NM-100	**0047**	**K (>0.1%), P**	**Zr**	**Ca, Na**

[$] ***Near 0.01%***

Overall remarks, attachments

Attached full study report

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

Remarks Dispersion protocol

Attached document D4.3_MinChemComposition: **ENV/JM/MONO(2015)17/ANN5**

Remarks Data in the report and detailes porocol in annex

Applicant's summary and conclusion

Cross-reference to other study

http://www.nanogenotox.eu/: **ENV/JM/MONO(2015)17/ANN22**

4.24 Agglomeration/aggregation

Endpoint study record: Agglomeration/aggregation_by by University of Graz

Administrative Data

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

Data source

Data access

other: performed and provided by Uni Graz

Materials and methods

Methods

other: PCS

Details on methods and data evaluation

As solid particles show low stability and a high tendency to aggregate in aqueous dispersions, our first goal was to produce a stable 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 baht) 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.

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

Sponsorship Program: Titanium Dioxide Report

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

Results of the particle characterization of NM100 in different biological media

NM100	**0,4 mg/ml TiO2 particles (NM100, pigment, TIONA) untreated**					
Medium	**Size (d.nm)**	**Z-Average (d.nm)**	**PdI**	**Zeta Potential (mV)**	**monomodal**	**Zeta Deviation**
MQ Wasser	391,2*/4862	343	0,176	-34		8,26
PBS	1440*/5236	2289	0,355	-36,5		6,81
DMEM + L-Glutamine	995,5	2129	0,662	-0,839*/-56,5/50,2	-7,62	49,8
DMEM + 1% FBS	736	606,8	0,168	-8,7		16,6
DMEM + 5% FBS	845,4	621,9	0,231	-9,95		15,3
DMEM + 10% FBS	639,1*/4763	582,4	0,262	-7,89		20,9
0,4 mg/ml TiO2 particles (NM100, pigment, TIONA) 1 min Sonifier (40% amplitude)						

Medium	Size (d.nm)	Z-Average (d.nm)	PdI	Zeta Potential (mV)	monomodal	Zeta Deviation
MQ Wasser	259,3	201,3	0,205	-24,5		10,5
PBS	2116	1624	0,219	-26,7	-27,6	161
DMEM + L-Glutamine	2973	2514	0,332	20,5*/-26/95,4	1,58	35,5
DMEM + 1% FBS	405,3	310,4	0,207	-9,14*/140	-10	41,6
DMEM + 5% FBS	408,8	315,2	0,194	107*/35,4/-21,2	1,75	275
DMEM + 10% FBS	345,8	283,9	0,176	78,4*/12,2/-27,1	-4,19	193

0,4 mg/ml TiO2 particles (NM100, pigment, TIONA) 20 min US-bath						
Medium	Size (d.nm)	Z-Average (d.nm)	PdI	Zeta Potential (mV)	monomodal	Zeta Deviation
MQ Wasser	378,8	307,6	0,199	-40,6		6,58
PBS	1042*/5236	1217	0,317	-20,2		16,7
DMEM + L-Glutamine	1059	1754	0,515	-1,55	-1,64	25,4
DMEM + 1% FBS	631,9*/5059	540,2	0,195	-11,4		16,2
DMEM + 5% FBS	522,7*/5017	450,4	0,223	-10,4		16,9
DMEM + 10% FBS	565,7	473,1	0,204	-11,3	-10,2	14,3

Results of the particle characterization with a Mastersizer 2000

NM100	TiO2 Partikel (NM100, pigment, TIONA)			
Medium	Size(0.1) [nm]	Size(0.5) [nm]	Size(0.9) [nm]	Sonification period
MQ Wasser	123	735	856241	0 min US
MQ Wasser	70	135	311	1min US
MQ Wasser	70	129	271	2 min US
MQ Wasser	89	255	7098	3 min US
MQ Wasser	89	249	4719	4 min US
MQ Wasser	90	262	10767	5 min US

Endpoint study record: Agglomeration/aggregation by University Applied Science of Hamburg (HAW Hamburg)

Administrative Data

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

Data source

Reference

Reference type publication

Author Judith Angelstorf Year 2013
Title PhD thesis, in preparation
Bibliographic source
Testing laboratory HAW Hamburg, TUHH Hamburg
Report no.
Owner company
Company study no.

Report date

Data access

other: performed and provided by HAW Hamburg

Cross-reference to same study

Angelstorf, J.S.1, Ahlf, W. 2; and Heise, S. 1 unpublished data.
1) University of applied scienes Hamburg, Lohbrügger Kirchstraße 65, 21033 Hamburg
2) Technical University of Hamburg-Harburg (TUHH)
Contact: Judith.angelstorf@haw-hamburg

Materials and methods

Methods

DLS

Details on methods and data evaluateon

For every concentration, 3 independently dispersed suspensions have been measured 10 times by DLS (Malvern Zetasizer). Mean particle sizes (reported as mean intensities in d.nm) and standard deviation (SD) were calculated for each concentration (n= 3*10=30).

Used **Protocols**

• Description of the test item: TiO2 particles dispersed in ultrapure water without any additives
• Application to test system: dispersion
• Description of the test media: M9 Media (6 g/L of Na2HPO4; 3 g/L of KH2PO4; 5 g/L of NaCl; 0,25 g/L of MgSO4•7H2O) dissolved in ultrapure water, Escherichia coli bacteria (density: 1000 FAU)
• Dispersion/sample preparation protocol (e.g. concentration, duration, method, energy input)
1. Stock suspension (400 mg/L):
4 mg of TiO2 are dispersed in 10 mL of ultrapure water in glas jars according to the following protocol:
1) 1 min magnetic stirring, 900 rpm
2) 5 min ultrasonic bath
2. Test suspensions
10 min after dispersion of the stock suspension, test suspensions are prepared by dilution with ultrapure water and dispersed according the same protocol:
1) 1 min magnetic stirring, 900 rpm
2) 5 min ultrasonic bath
Test suspensions are added to the test vessels 10 min after dispersion. Concentration of testsuspensions = 2 x test concentration, in the test vessels suspensions are diluted with the food media (50:50). Following concentrations were applied:

Data gathering

Test materials

State of test material

Dispersion

Results and discussions

Aggregation Index

Remarks on results including tables and figures

see attachment

Overall remarks, attachments

Overall remarks

Results show an increase in mean particle size and variation (SD) with increasing concentrations. Addition of the test media M9 leads to further agglomeration.

Tab. 1 Mean particle sizes [d.nm] shown as mean intensities and SD, Poly Dispersity Index (PDI) for suspensions of P25 in ultrapure water before added to the test media

Suspension		$bTiO_2$		
Conc. [mg/l]	Media	PDI	Mean size [nm]	SD [nm]
2	H_2O	0,31	296	97
6	H_2O	0,24	326	69
40	H_2O	0,19	331	46
60	H_2O	0,18	335	36
200	H_2O	0,18	357	39

2) in test media

Tab. 1 Mean particle sizes [d.nm] shown as mean intensities and SD, Poly Dispersity Index (PDI) for suspensions of P25 in ultrapure water before added to the test media

Suspension		$bTiO_2$		
Conc. [mg/l]	Media	PDI	Mean size [nm]	SD [nm]
1	M9	0,46	306	90
3	M9	0,31	361	46
10	M9	0,26	516	92
30	M9	0,23	735	80
100	M9	0,24	1509	177

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

Reference type	study report		
Author	KA Jensen	**Year**	2013
Title	Deliverable 4.3: Crystallite size, mineralogical and chemical purity of NANOGENOTOX nanomaterials		
Bibliographic source			
Testing laboratory	NRCWE (DK) and IMC-BAS (BG) , LNE	**Report no.**	4.3
Owner company			
Company study no.		**Report date**	

Data access

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 acharacteristic pattern, which is unique for each material. XRD can therefore be used to identifydifferent 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 limitationsAs any method, sizing of crystallites by XRD has limitations. Most importantly, the method has both upper and lower limits, where the lower limit is very much material dependent. Large crystals have narrow reflections, and as rule of thumb, sizes cannot be calculated for crystals larger than 100 nm.As an example, using the first reflection from Anatase as

starting point, and using theScherrer 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 listthose as results; however for the comparison the numbers have been listed with one decimal.The real and important question is however; how accurate are the calculations? It is known that the larger the crystals get, the more the instrument contribution matters. However for very small crystals it is difficult to find the background and thereby the height of the reflection, so in this case it is also difficult to find the right FWHM, and calculate the right size. It was assumed that the results are more uncertain than we have listed. Our estimate is that the uncertainty probably is on the order of ±5 nm for all the samples.

Details on methods and data evaluation

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

Data gathering

Instruments

The data from NRCWE were measured at room temperature (25°C) on a Bruker D8 Advanceddiffractometer in reflection mode with Bragg-Brentano geometry. The analysis were made usingCuKα1 X-rays (1.5406 Å) generated using a sealed Cu X-ray tube run at 40 kV and 40 mA. The x-raybeam was filtered for CuKα2 and focused using a primary beam Ge monochromator and fixeddivergence slit 0.2°. The analyses were made in the stepping mode stepping 0.02 degree 2theta persecond 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 $K_{\alpha 1}$ and $K_{\alpha 2}$ rays. For data comparison, the $K_{\alpha 2}$ contribution was therefore stripped from the data using the EVA software (Bruker).

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

Calibration

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

Reproducibility

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM-100

State of test material

other: fluffy powder

Confidential details on test material

Commercial name: Tiona AT-1

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 100 is anatase

Remarks on results including tables and figures

X-ray diffractograms shows good agreement between the results from IMC-BAS and NRCWE.

The Anatase samples, NM100, NM101, NM102 and NM105

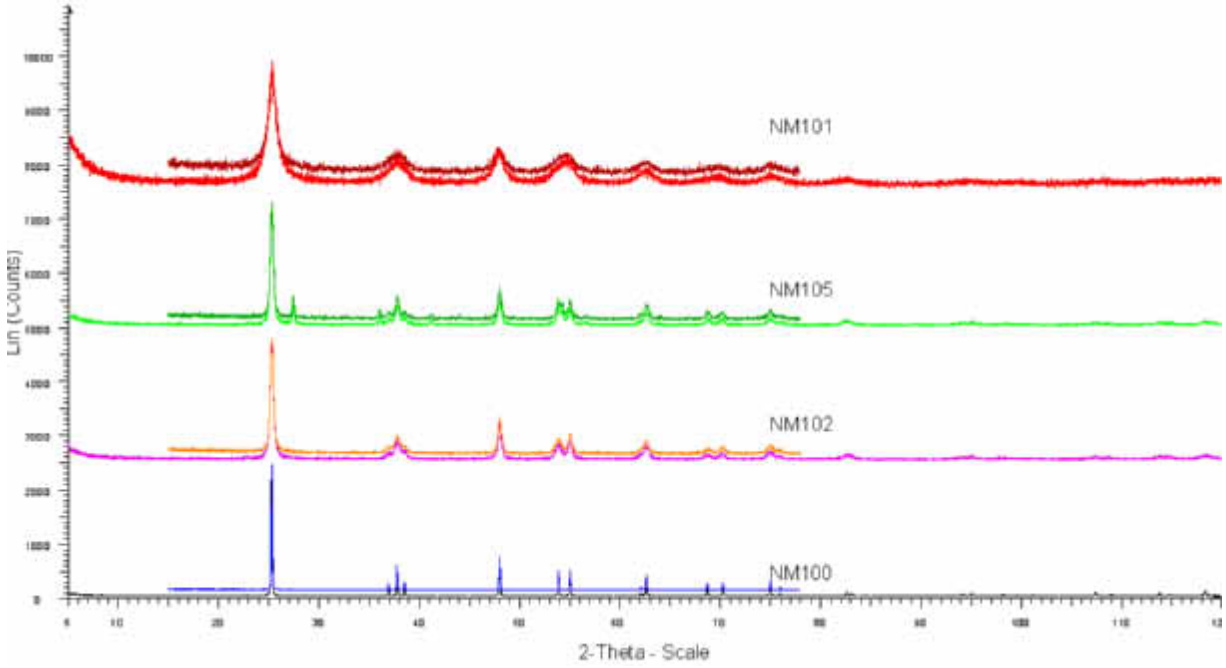


Figure 3-3 The diffraction data from NRCWE and IMC-BAS. Overall the data are very alike. The long measurements are done at IMC-BAS and the short ones at NRCWE.

Table 3-5 Crystallite sizes (nm) determined from measurements on NM100, Anatase

Vial	LNE	IMC-BAS			NRCWE		
	Scherrer Equation	Peak fit, FWHM vs standard	Topas 4.2, standard less	Fullprof, quartz standard	Scherrer Equation	Topas 4.1, IB	Topas 4.1, FWHM
0006+0007					> 100	> 100	> 100
0016	141.2						
0079		56.66€	61.87€ (2.23)	168.18€ (1.9)			
0081							
0083							
0211+0213+0214					> 100	> 100	> 100
0406+0408					> 100	> 100	> 100

€ ***Average of three samples.***

Overall remarks, attachments

Attached full study report

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

Remarks Dispersion protocol

Attached document D4.3_MinChemComposition: **ENV/JM/MONO(2015)17/ANN5**

Remarks Data in the report and detailes 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

http://www.nanogenotox.eu/ : **ENV/JM/MONO(2015)17/ANN22**

4.26 Crystallite and grain size

4.27 Aspect ratio/shape

4.28 Specific surface area

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

Reference

Reference type	study report		
Author	KA Jensen	**Year**	2013
Title	Deliverable 4.4: Determination of specific surface area of NANOGENOTOX nanomaterials		
Bibliographic source			
Testing laboratory	IMC-BAS (BG)	**Report no.**	D4.4
Owner company			
Company study no.		**Report date**	

Data access

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, formationand potential applications of different natural materials. For this reason it is important to determineand control them accurately. The most widely used technique for estimating surface area is the socalled BET method (Brünauer, Emmett and Teller, 1938) [5]. The concept of the theory is anextension of the Langmuir theory, which is a theory for monolayer molecular adsorption, tomultilayer 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 Langmuirtheory 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 theadsorbate (at temperature of liquid Nitrogen). As adsorption or desorption occurs the pressure in thesample cell changes until equilibrium is established. The quantity of gas adsorbed or desorbed at theequilibrium pressure is the difference between the amount of gas admitted or removed and theamount required to fill the space around the adsorbent (void space).Smaple preparationno dpeciall treatment needed. Measurments performed on powder. 0.1 g of the material placed it in the appropriate cell size (the volume of the sample mayvary from sample to sample due to difference in density etc.).Details of the method and values of used paramters 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-100

State of test material

other: fluffy powder

Confidential details on test material

Commercial name: Tiona AT-1

Results and discussions

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

Material	BET surface	Total pore volume	Micro surface area	Micropore volume
	m^2/g	ml/g	m^2/g	ml/g
NM100	**9.230**	**0.0324**	**0.0**	**0.0**

Remarks on results including tables and figures

Total pore volume (mL/g): 0.0324Micropore volume (mL/g): 0.0

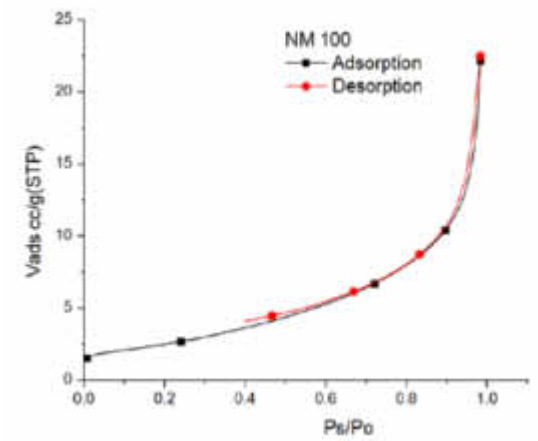


Figure 4.10: Isotherms of nitrogen sorption experiments at 77K for the TiO_2 nanomaterials.

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. 4_specific_surface_area: **ENV/JM/MONO(2015)17/ANN2**

Remarks Data in the report and detailes porocol in annex

Applicant's summary and conclusion

Cross-reference to other study

http://www.nanogenotox.eu/ : **ENV/JM/MONO(2015)17/ANN22**

4.29 Zeta potential

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

Titel: 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).

Overall remarks, attachments

Attached background material

Results of the particle characterization of NM100 in different biological media

NM100	**0,4 mg/ml TiO2 particles (NM100, pigment, TIONA) untreated**					
Medium	**Size (d.nm)**	**Z-Average (d.nm)**	**PdI**	**Zeta Potential (mV)**	**monomodal**	**Zeta Deviation**
MQ Wasser	391,2*/4862	343	0,176	-34		8,26
PBS	1440*/5236	2289	0,355	-36,5		6,81
DMEM + L-Glutamine	995,5	2129	0,662	-0,839*/-56,5/50,2	-7,62	49,8
DMEM + 1% FBS	736	606,8	0,168	-8,7		16,6
DMEM + 5% FBS	845,4	621,9	0,231	-9,95		15,3
DMEM + 10% FBS	639,1*/4763	582,4	0,262	-7,89		20,9
0,4 mg/ml TiO2 particles (NM100, pigment, TIONA) 1 min Sonifier (40% amplitude)						
Medium	**Size (d.nm)**	**Z-Average (d.nm)**	**PdI**	**Zeta Potential (mV)**	**monomodal**	**Zeta Deviation**
MQ Wasser	259,3	201,3	0,205	-24,5		10,5
PBS	2116	1624	0,219	-26,7	-27,6	161
DMEM + L-Glutamine	2973	2514	0,332	20,5*/-26/95,4	1,58	35,5
DMEM + 1% FBS	405,3	310,4	0,207	-9,14*/140	-10	41,6
DMEM + 5% FBS	408,8	315,2	0,194	107*/35,4/-21,2	1,75	275
DMEM + 10% FBS	345,8	283,9	0,176	78,4*/12,2/-27,1	-4,19	193

0,4 mg/ml TiO2 particles (NM100, pigment, TIONA) 20 min US-bath						
Medium	**Size (d.nm)**	**Z-Average (d.nm)**	**PdI**	**Zeta Potential (mV)**	**monomodal**	**Zeta Deviation**
MQ Wasser	378,8	307,6	0,199	-40,6		6,58
PBS	1042*/5236	1217	0,317	-20,2		16,7
DMEM + L-Glutamine	1059	1754	0,515	-1,55	-1,64	25,4
DMEM + 1% FBS	631,9*/5059	540,2	0,195	-11,4		16,2
DMEM + 5% FBS	522,7*/5017	450,4	0,223	-10,4		16,9
DMEM + 10% FBS	565,7	473,1	0,204	-11,3	-10,2	14,3

Results of the particle characterization with a Mastersizer 2000

NM100	**TiO2 Partikel (NM100, pigment, TIONA)**			
Medium	**Size(0.1) [nm]**	**Size(0.5) [nm]**	**Size(0.9) [nm]**	**Sonification period**
MQ Wasser	123	735	856241	0 min US
MQ Wasser	70	135	311	1min US
MQ Wasser	70	129	271	2 min US
MQ Wasser	89	255	7098	3 min US
MQ Wasser	89	249	4719	4 min US
MQ Wasser	90	262	10767	5 min US

Endpoint study record: Zeta potential by University of Applied Science Hamburg (HAW Hamburg)

Administrative Data

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

Data source

Reference

Reference type publication
Author Judith Angelstorf **Year** 2013
Title PhD thesis, in preparation
Bibliographic source
Testing laboratory HAW Hamburg, TUHH Hamburg
Report no.
Owner company
Company study no.

Report date

Data access

other: performed and provided by HAW Hamburg

Cross-reference to same study

Angelstorf, J.S.1, Ahlf, W. 2; and Heise, S. 1 unpublished data.
1) University of applied scienes Hamburg, Lohbrügger Kirchstraße 65, 21033 Hamburg
2) Technical University of Hamburg-Harburg (TUHH)
Contact: Judith.angelstorf@haw-hamburg

Materials and methods

Methods

other: DLS

Used Protocols

• Description of the test item: TiO2 particles dispersed in ultrapure water without any additives
• Application to test system: dispersion
• Description of the test media: M9 Media (6 g/L of Na2HPO4; 3 g/L of KH2PO4; 5 g/L of NaCl; 0,25 g/L of MgSO4•7H2O) dissolved in ultrapure water, Escherichia coli bacteria (density: 1000 FAU)
• Dispersion/sample preparation protocol (e.g. concentration, duration, method, energy input)
1. Stock suspension (400 mg/L):
4 mg of TiO2 are dispersed in 10 mL of ultrapure water in glas jars according to the following protocol:
1) 1 min magnetic stirring, 900 rpm
2) 5 min ultrasonic bath
2. Test suspensions
10 min after dispersion of the stock suspension, test suspensions are prepared by dilution with ultrapure water and dispersed according the same protocol:
1) 1 min magnetic stirring, 900 rpm
2) 5 min ultrasonic bath
Test suspensions are added to the test vessels 10 min after dispersion. Concentration of testsuspensions = 2 x test concentration, in the test vessels suspensions are diluted with the food media (50:50). Following concentrations were applied:

Data gathering

Test materials

State of test material

dispersion

Results and discussions

In medium (specify)

Zeta-Potentials were measured three times for every concentration. Mean Zeta-Potential for ultrapure water: -48.42+/-2.2mV M9 test media:-28.54+/-3.4mV

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 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

0.099ml/mg

4.31 Dustiness

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

Reference type	study report		
Author	KA Jensen	**Year**	2013
Title	Deliverable 4.4: Determination of specific surface area of NANOGENOTOX nanomaterials		
Bibliographic source			
Testing laboratory	NRCWE (DK)	**Report no.**	D4.4
Owner company			
Company study no.		**Report date**	

Data access

owner: NANOGENOTOX

Data protection claimed

yes, but willing to share

Materials and methods

Methods

BET

Principles of method if other than guideline

Surface area and porosity are important characteristics, in understanding the structure, formationand potential applications of different natural materials. For this reason it is important to determineand control them accurately. The most widely used technique for estimating surface area is the socalled BET method (Brünauer, Emmett and Teller, 1938) [5]. The concept of the theory is anextension of the Langmuir theory, which is a theory for monolayer molecular adsorption, tomultilayer 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 Langmuirtheory 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 theadsorbate (at temperature of liquid Nitrogen). As adsorption or desorption occurs the pressure in thesample cell changes until equilibrium is established. The quantity of gas adsorbed or desorbed at theequilibrium pressure is the difference between the amount of gas admitted or removed and theamount required to fill the space around the adsorbent (void space).

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

double test

Test materials

Test material equivalent to submission substance identity

yes

Reference Material/Nanomaterial and Sample identification number

Identifier Reference Material/Nanomaterial

Identity NM-100

Test material identity

Identifier CAS number

Identity 7631-86-9

State of test material

other: fluffy powder

Confidential details on test material

Commercial name: Tiona AT-1 (Cristal Global)

Results and discussions

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

Material	BET surface	Total pore volume	Micro surface area	Micropore volume
	m^2/g	ml/g	m^2/g	ml/g
NM100	9.230	0.0324	0.0	0.0

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. 4_specific_surface_area: **ENV/JM/MONO(2015)17/ANN2**

Remarks Data in the report and detailes porocol in annex

Applicant's summary and conclusion

Cross-reference to other study

http://www.nanogenotox.eu/: **ENV/JM/MONO(2015)17/ANN22**

4.33 Pour density

4.34 Photocatalytic activity

4.35 Radical formation potential

4.36 Catalytic activity

5. ENVIRONMENTAL FATE AND PATHWAYS

5.1 Stability

5.1.1 Phototransformation in air

5.1.2 Hydrolysis

5.1.3 Phototransformation in water

5.1.4 Phototransformation in soil

5.1.5 Preliminary: Dispersion stability in water

Endpoint study record: Dispersion stability in water by University of Vienna,

Administrative Data

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

Conclusions

von der Kammer,F., Hofmann, T. (2012), Testing the OECD selected alternative nano-TiO2 materials for dispersion stability, environmental behaviour and fate. Project report, University of Vienna, Department of Environmental Geosciences Test material: P25, Hombikat UV100, UV-Titan M212, UV-Titan M262, PC-105, Tiona AT-1 Source type: Project report Guideline: --- Subject: Dispersion stability and

environmental fate of P25 compared to alternative materials Test media + conditions: In the proposed test system a stable dispersion of the to-be-tested particles is separated into 300-450 subsamples and each is then subjected to a different hydrochemical condition. This results in a three dimensional matrix of dispersion stability over pH and ion concentration. By applying different salts as NaCl, CaCl2 etc. a set of matrices is obtained, the results become multi-dimensional. Study type: laboratory test Test duration: 12-15h Application method: Weighing of 50/100/250mg in 1L MQ water, adjusting pH to 7-7.5 with either 1 mol/L HCl or NaOH, 30 sec. ultrasonic bath treatment, followed by a wetting time of 24 hours. Using ultrasonic bath to disperse the particles (120W output, constant, 60 min.), adjusting pH to 7-7.5 as before. In the described test a stable dispersion of TiO2 in ultrapure water is subjected to a change in water chemistry and the phase separation (aggregation and settling of the particles) is measured once after a given time period. Endpoint: TiO2 nanoparticle concentration, particle size and electrophoretic mobility. Chemical analysis, Material characterization: The concentration of TiO2 in the supernatant was determined by measuring the nephelometric turbidity (Hach 2100N IS Turbiditymeter, 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 1: Project report TiO2 OECD BMVIT.pdf (page 50 - 75): ENV/JM/MONO(2015)17/ANN13

6. ECOTOXICOLOGICAL INFORMATION

6.1 Aquatic toxicity

6.1.1 Short-term toxicity to fish

Endpoint study record: Short-term toxicity to fish.001by RWTH-Aachen University

Administrative Data

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

Data source

Reference

Reference type study report
Author Anne Wyrwoll
Title
Bibliographic source
Testing laboratory Institute for Environmental Research, RWTH-Aachen University, Germany
Owner company Federal Environment Agency Germany, Institute for Environmental Research, RWTH-Aachen University, Germany
Report no.
Company study no.
Report date

Data access

data submitter is data owner

Cross-reference to same study

The same study was performed with the nanomaterials NM 101 and NM 102.

Materials and methods

Test guideline

Qualifier according to
Guideline other guideline: Fish Embryo Toxicity
Deviations yes

Principles of method if other than guideline

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

GLP compliance

no in the style of GLP

Test materials

Analytical monitoring

No

Test organisms

Test organisms (species)

Danio rerio

Details on test organisms

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

Study design

Test type

Static

Water media type

freshwater

Limit test

yes

Total exposure duration

72 h Remarks

Test conditions

Test temperature

26 °C

Reference substance (positive control)

yes 3,4-dichloroaniline

Results and discussions

Effect concentrations

Duration	72 h
Endpoint	NOEC
Effect conc.	>= 100 mg/L
Nominal/Measured	nominal
Conc. based on	
Basis for effect	mortality
Remarks (e.g. 95% CL)	
Duration	**72 h**
Endpoint	**LOEC**
Effect conc.	**> 100 mg/L**
Nominal/Measured	**nominal**
Conc. based on	
Basis for effect	**mortality**
Remarks (e.g. 95% CL)	

Details on results

NM 100 did not show any effect on the mortality or hatching rate of D. rerio within an exposure period of 72 h.

Results with reference substance (positive control)

80% mortality was observed for the positive controls after an exposure period of 72 h. This result seems to be in line with the criteria given in the guideline (4 mg/L exposure results in a minimum mortality of 30% after an exposure period of 96 h).

Overall remarks, attachments

Attached background material

Attached document 2: SOP Preparation of a NM 100 suspension.pdf (page 76 - 78): ENV/JM/MONO(2015)17/PART2/ANN1

Applicant's summary and conclusion

Validity criteria fulfilled

Yes

Conclusions

NM 100 had no effect on the mortality of D. rerio embryos up to a concentration of 100 mg/L under the conditions tested. As nano-TiO2 is known to be photoactived by illumination with solar radiation, it stays unclear whether NM 100 would induce toxic effects on D. rerio during a parallel exposure to solar radiation. This was not examined in the present study, but should be considered in further studies.

Endpoint study record: zebrafish by INIA

Administrative Data

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

Data source

Data access

other: performed and provided by INIA, Spain

Materials and methods

Principles of method if other than guideline

Toxicity tests were conducted in accordance with the OECD TG 212 Fish, short-term toxicity test on embryo and sac fry stages

Test materials

Details on test material

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

Details on 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

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

Effect concentrations

Duration	8 d
Endpoint	LOEC
Effect conc.	100 mg/L
Nominal/Measured	nominal
Conc. based on	
Basis for effect	other: growth
Remarks (e.g. 95% CL)	

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 100 mg/l.

Results with reference substance (positive control)

control group receiving only embryo medium

Reported statistics and error estimates

Body length measurements were compared using one way ANOVA followed by post hoc Dunnett's tes. 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.

Overall remarks, attachments

***Acute Toxicity to Fish**

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

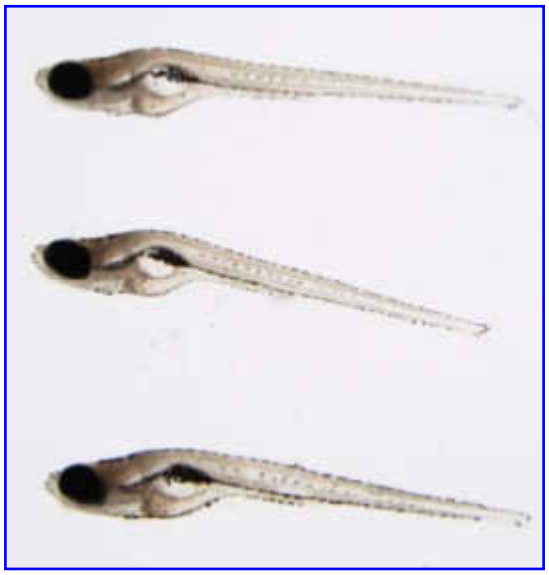
Date: 21.06.12

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

TREATMENT	Exp(mg/l)	HATCHED	DEAD	SURVIVORS	% survival
CONTROL	**A**	10			
CONTROL	**B**	7		17.00	100
NM 101	**10 A**	7			
	10 B	6		13.00	100
	100 A	8			
	100 B	7		15.00	100
	1000 A	9			
	1000 B	7		16.00	100
NM 103	**10 A**	6			
	10 B	8		14.00	100
	100 A				
	100 B	9	1	8.00	88.89
	1000 A	15			
	1000 B	6		21.00	100
NM 104	**10 A**	8			
	10 B	8		16.00	100
	100 A	8			
	100 B	7		15.00	100
	1000 A	8			
	1000 B	2		10.00	100
NM 105	**10 A**	6			
	10 B	8		14.00	100
	100 A	9			

	100 B	9		18.00	100
	1000 A	7			
	1000 B	8		15.00	100
P-25 Evonik	**10 A**	9			
	10 B	9		18.00	100
	100 A	8			
	100 B	5		13.00	100
	1000 A	9			
	1000 B	10		19.00	100
P-25 Evonik	**10 A**	7			
	10 B	9		16.00	100
	100 A	8			
	100 B	7		15.00	100
	1000 A	7			
	1000 B	8	1	14.00	93.33
P-25 Evonik	**10 A**	9			
	10 B	8		17.00	100
	100 A	8			
	100 B	8		16.00	100
	1000 A	8			
	1000 B	8		16.00	100

Ctr



Treatment P-25 EVONIK -1000

Treatment NM 101-1000

Treatment NM 103-1000

Treatment NM 104-1000

Treatment NM 105-1000

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

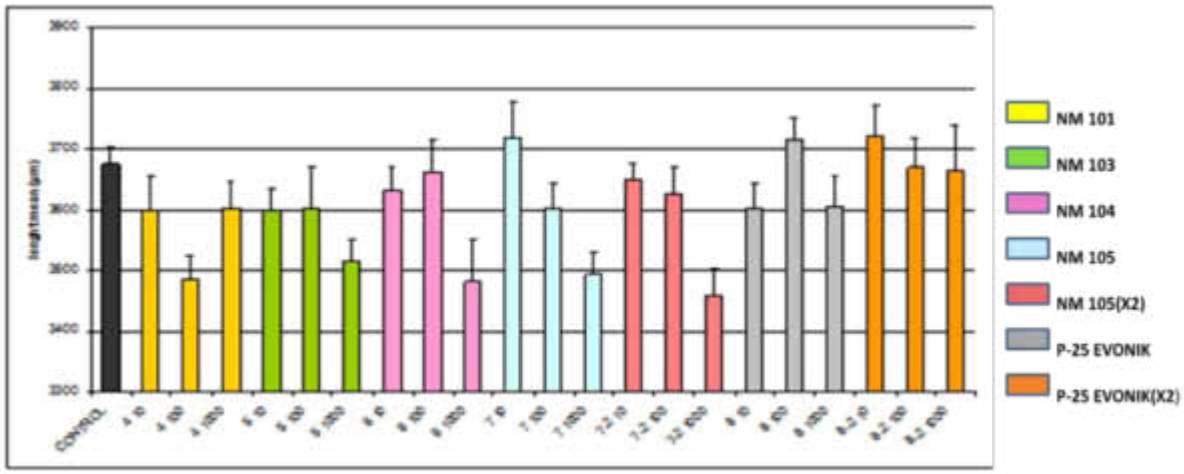


Fig 3. Mean ± SE body length of 8 dpf larvae exposed to the studied NM at 10, 100 and 1000 mg/L concentrations.

6.1.2 Long-term toxicity to fish

6.1.3 Short-term toxicity to aquatic invertebrates

Endpoint study record: Short-term toxicity to aquatic invertebrates.001by RWTH Aachen

Administrative Data

Purpose flag key study ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Study period January 2013 - April 2013
Reliability 1 (reliable without restriction)
Rationale for reliability in the style of GLP

Data source

Reference

Reference type study report
Author Anne Wyrwoll
Title
Bibliographic source
Testing laboratory RWTH Aachen
Owner company UBA, RWTH Aachen

Data access

data submitter is data owner

Materials and methods

Test guideline

Qualifier according to
Guideline OECD Guideline 202 (Daphnia sp. Acute Immobilisation Test)
Deviations no

Principles of method if other than guideline

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

GLP compliance

No

Test organisms

Test organisms (species)

Daphnia magna

Details on test organisms

< 24 h old neonates were used as test organisms.

Study design

Test type

static

Water media type

freshwater

Total exposure duration

48 h Remarks

Test conditions

Test temperature

19-20 °C

pH
6.61-7.84 The variation of the pH in the test medium between the test initiation and termination in each test was not greater than 1.5 units

Dissolved oxygen
7.39-8.8 mg/L

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

Results and discussions

Effect concentrations

Duration	48 h		
Endpoint	NOEC		
Effect conc.	> 50 mg/L		
Nominal/Measured	nominal		
Conc. based on	test mat.	**Basis for effect**	mobility ; Results of the laboratory light test.
Remarks (e.g. 95% CL)			

Duration	48 h		
Endpoint	EC50		
Effect conc.	3.88 mg/L		
Nominal/Measured	nominal		
Conc. based on	test mat.	Basis for effect	mobility ; Results of the simulated solar radiation test.
Remarks (e.g. 95% CL)	95%-CL 0.16-40.73 mg/L (lower/upper)		

Duration	48 h		
Endpoint	NOEC		
Effect conc.	1.85 mg/L		
Nominal/Measured	nominal		
Conc. based on	test mat.	**Basis for effect**	mobility ; Results of the simulated solar radiation test.
Remarks (e.g. 95% CL)			

Duration	48 h		
Endpoint	LOEC		
Effect conc.	5.56 mg/L		
Nominal/Measured	nominal		
Conc. based on	test mat.	**Basis for effect**	mobility ; Results of the simulated solar radiation test.
Remarks (e.g. 95% CL)			

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

Overall remarks, attachments

Attached document 2: SOP Preparation of a NM 100 suspension.pdf (page 76 - 78): ENV/JM/MONO(2015)17/ANN1

Applicant's summary and conclusion

Validity criteria fulfilled

Yes

Conclusions

The results of the present study show that toxicity of NM 100 to D. magna was promoted by environmental realistic levels of SSR. It is suggested that SSR induced toxicity is a consequence of SSR induced ROS production through the TiO2 material. This study emphasizes the need for testing nano-TiO2 under environmental realistic levels of SSR in ecotoxicity assays because SSR induced ROS production seems to be a main mechanism of TiO2 toxicity.

6.1.4 Long-term toxicity to aquatic invertebrates

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

Data access

other: performed and provided by University of Victoria, Canada

Cross-reference to same study

Hammond, SA. Evaluation of the Effects of Nanometals on Cultured Rana catesbeiana Tail-fin Tissue. 2011. Honours Thesis in partial fulfillment of the requirements for the degree of Bachelor of Science (Hon). Department of Biochemistry and Microbiology. University of Victoria, BC, Canada.

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 Tiona at 80 mg/L: 681.1nm
in dH2O:

DLS effective diameter of non-sonicated Tiona at 80 mg/L: 303.3nm
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 Tiona at 80 mg/L: 9.20mV
in dH2O:
Zeta potential of non-sonicated Tiona at 80 mg/L: -41.46mV

Details on sampling

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: Powder added to dH2O and vortexed to disperse Diluted to 1000x test concentrations in dH2O 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

o In brief: Eight 6 mm biopsies were collected from each of eight Rana catesbiana tadpoles, from the dorsal and ventral tailfins. 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 TiO2 ± thyroid hormone (TH). NaOH is the vehicle for TH and was therefore included in all treatments. 1000x stocks of TiO2 were prepared in dH2O 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 TiO2 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 TiO2 containing medium before addition of NaOH or TH in NaOH Positive and negative control groups and treatment negative control for TiO2 (no TH): NaOH only negative control for TiO2 + TH: TH in NaOH only Solvent: for TiO2: dH2O for TH: NaOH in dH2O 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

In the absence of TH at 80 ng/L TiO2, TRb transcript abundance was decreased by 1.4-fold, and RLK1 was decreased 1.6-fold (p-value = 0.019, p-value = 0.044 respectively). In the TH-treated samples, 8 ng/L TiO2 increased Hsp30 expression by 1.6-fold over the TH-only control (p-value = 0.034). n the absence of TH, the medium dose lowered expression of TRb and RLK1, whereas the addition of TH ameliorated these effects; this suggests that the presence of TH has a greater effect on these transcripts than the TiO¬2 particles¬¬. TRb and RLK1 transcript levels are TH-responsive, and perturbations in their expression are suggestive of TH-signalling disruption. Hsp30 expression was increased in the low dose treatment with TH, but no dose-responsive trend was observed. HSP30 is not directly TH-responsive, and an increase in the steady-state levels of its mRNA is associated with cellular stress.

Remarks on results including tables and figures

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

n the absence of TH, the medium dose lowered expression of TRb and RLK1, whereas the addition of TH ameliorated these effects; this suggests that the presence of TH has a greater effect on these transcripts than the TiO¬2 particles¬¬. TRb and RLK1 transcript levels are TH-responsive, and perturbations in their expression are suggestive of TH-signalling disruption. Hsp30 expression was increased in the low dose treatment with TH, but no dose-responsive trend was observed. HSP30 is not directly TH-responsive, and an increase in the steady-state levels of its mRNA is associated with cellular stress.

Cross-reference to other study

Hinther, A., Domanski, D., Vawda, S. & Helbing, C.C. C-fin: a cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals. Environ Toxicol Chem 29, 380-8 (2010). Taylor, A.C. & Kollros, J.J. Stages in the normal development of Rana pipiens larvae. Anatomical Record 94, 7-24 (1946). Bustin, S.A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55, 611-22 (2009).

Endpoint study record: Activated sewage sludge by RWTH-Aachen

Administrative Data

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

Data source

Reference

Reference type study report
Author Anne Wyrwoll
Testing laboratory Institute for Environmental Research, RWTH-Aachen University, Germany
Owner company Federal Environment Agency Germany; Institute for Environmental Research, RWTH-Aachen University, Germany

Data access

data submitter is data owner

Cross-reference to same study

The same study was performed with the nanomaterials NM 102 and NM 101.

Materials and methods

Test guideline

Qualifier according to
Guideline other guideline: OECD 209 Activated sludge, respiration inhibition test

GLP compliance

no in the style of GLP

Test materials

Analytical monitoring

No

Test organisms

Test organisms (species)

other: Activated sludge

Details on test organisms

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

Study design

Test type

static

Water media type

Freshwater

Limit test

No

Total exposure duration

3h

Test conditions

Reference substance (positive control)

yes 3,5-dichlorophenol

Any other information on materials and methods incl. tables

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

Results and discussions

Effect concentrations

Duration	3 h
Endpoint	LOEC
Effect conc.	> 1000 mg/L
Nominal/Measured	nominal
Conc. based on	
Basis for effect	other: respiration
Remarks (e.g. 95% CL)	
Duration	3 h
Endpoint	NOEC
Effect conc.	>= 1000 mg/L
Nominal/Measured	nominal
Conc. based on	
Basis for effect	other: respiration
Remarks (e.g. 95% CL)	

Details on results

The controls fulfilled the validity criteria of the guideline. NM 100 did not affect the heterotrophic activated sludge respiration nor the nitrification, therefore no effective concentrations (ECx) were calculated. The lowest observed effect concentration is higher and the no observed effect concentration is equal to or higher than 1000 mg/L.

Results with reference substance (positive control)

3,5-Dichlorophenol was used as reference substance (3.2, 10 and 32 mg/L). The EC50 accounted to 11.2 mg/L and was within the range stated in the OECD guideline (2-25 mg/L).

Overall remarks, attachments

Attached document 2: SOP Preparation of a NM 100 suspension.pdf (page 76 - 78): ENV/JM/MONO(2015)17/ANN1

Applicant's summary and conclusion

Validity criteria fulfilled

Yes

Conclusions

NM 100 did not affect the respiration of the activated sludge under the test conditions used.

6.2 Sediment toxicity

Endpoint study record: Sediment toxicity by University of Applied Science Hamburg(HAW Hamburg)

Administrative Data

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

Data source

Reference

Reference type publication
Author Judith Angelstorf **Year** 2013
Title Effekte von Titandioxidnanopartikeln auf den Nematoden Caenorhabditis elegans unter besonderer Berücksichtigung von UV-Strahlung
Bibliographic source HAW Hamburg, TUHH Hamburg
Testing laboratory HAW Hamburg, TUHH Hamburg

Data access

other: performed and provided by HAW Hamburg, Germany

Cross-reference to same study

Angelstorf, J.S.1, Ahlf, W. 2; and Heise, S. 1 unpublished data.
1) University of applied scienes Hamburg, Lohbrügger Kirchstraße 65, 21033 Hamburg
2) Technical University of Hamburg-Harburg (TUHH)
Contact: Judith.angelstorf@haw-hamburg

Materials and methods

Test guideline

Qualifier according to
Guideline other guideline: ISO 10872
Deviations yes Test vessels of glass

Principles of method if other than guideline

Study is performed according to the ISO 10872 test guideline with only negligible modifications (test vessels). For the preparation of TiO2 suspensions, a standard operation procedure (SOP) has been developed, which is followed strictly for all experiments realized. Nevertheless ISO 10872 is not designed for nanomaterial testing.

GLP compliance

yes

Test materials

Analytical monitoring

no

Details on sampling

1. Stock suspension (400 mg/L): 4 mg of TiO2 are dispersed in 10 mL of ultrapure water in glas jars according to the following protocol: 1) 1 min magnetic stirring, 900 rpm 2) 5 min ultrasonic bath, intensity: 2. Test suspensions 10 min after dispersion of the stock suspension, test suspensions are prepared by dilution with ultrapure water and dispersed according the same protocol: 1) 1 min magnetic stirring, 900 rpm 2) 5 min ultrasonic bath, intensity Test suspensions are added to the test vessels 10 min after dispersion. Concentration of test suspensions = 2 x test concentration, in the test vessels they are diluted with the food media (50:50). For every concentration, 6 independently dispersed suspensions have been measured 10 times by DLS (Malvern Zetasizer). Mean particle sizes (reported as mean intensities in d.nm) and standard deviation (SD) were calculated for each concentration (n= 6*10=60). Results show an increase in mean particle size and variation (SD) with increasing concentrations. Addition of the test media M9 leads to further agglomeration.

Details on sediment and application

water only

Test organisms

Test organisms (species)

Caenorhabditis elegans

Details on test organisms

Source of organisms: Caenorhabditis Genetics Center Strain: wild type strain N2 breeding method: maintained on agar plates consisting of Nematode Growth Media, with a bacterial lawn of E.coli OP 50 Age: 1. Stage Juvenils (J1), selected with a micropipette from filtrate (juveniles are separated from older organisms by using a filter cascade with a 5 µm and 10 µm gauze)

Study design

Study type

laboratory study

Test duration type

short-term toxicity

Test type

static

Total exposure duration

4 d

Remarks via food media

Test conditions

Test temperature

-20°C+/- 1°C, 18.5°C +/- 1.5°C during irradiation (30 min in SSR-test)

pH

6,75+/- 0,25

Salinity

0,055µS/cm

Nominal and measured concentrations

1, 3, 10, 30 and 100 mg/L

Details on test conditions

Test design: 4 replicates, 10 individuals per replicate, 5 concentrations:1, 3, 10, 30 and 100 mg/L, 2 treatments: 1)no-SSR: samples were incubated without simulated solar radiation (SSR); 2) SSR: samples

were irradiated with SSR for 30 minutes, 4 hours after start of the test with an intensity of 231 W/m2 at 300-800 nm. Substance is dispersed in ultrapure water and added to the food media directly before the addition of test organisms

Any other information on materials and methods incl. tables

Exposure vessel type: Duran® Glass vessels, volume: 20 mL, headspace 17.5 mL, sealed with Parafilm, no aeration. SSR test: samples were irradiated in Q-Sun Xe-B-1 with Chiller, Daylight Q filter, Q-Lab Deutschland GmbH

Results and discussions

Details on results

Particles agglomerate and settle to the bottom of the test vessel within the first 12 h. Organisms are located on the bottom of the vessel and exposed to the particles for the whole test period of 96 h. Statistical method: One way ANOVA at α= 0.05, post test Dunett, no significant differences observed- Biological observations: TiO2 particles are ingested by C.elegans and accumulate in the intestinal tract of the worms. So far, there is no evidence for further uptake into the tissue of the organism. Accumulated particles in the gut inhibit the uptake of fluorescent microparticles, simulating food particles. -Control performances: negative control (incubated without test substance with simulated solar radiation (SSR) (for SSR-test) and without SSR (for no-SSR Test). Validity criteria are met.

Results with reference substance (positive control)

Control performances: negative control (incubated without test substance with simulated solar radiation (SSR) (for SSR-test) and without SSR (for no-SSR Test). Validity criteria are met. No reference substance was used for this test, quality control of the test organisms is performed regularly with Benzylcetyldimethylammonium chloride monohydrate (BAC-C16)

Reported statistics and error estimates

Statistical method: One way ANOVA at α= 0.05, post test Dunett, p-values <0,01, 95% confidence limits: 11.61 to 55.52; reference 33.56

Overall remarks, attachments

Overall remarks

Does not inhibit reproduction and growth of C.elegans at all tested concentrations. SSR not evoke any effects. Inhibition of feeding by blocking the defecation

Attached background material

sediment species-1_NM100_J Angelstorf_HAW Hamburg.docx: ENV/JM/MONO(2015)17/ANN18

Results
ENV/JM/MONO(2015)17/PART2/ANN2

Unit: mg/L

Concentration type: Endpoint value is based on nominal concentration

Measured concentrations

NM100	
Nominal Conc. (mg/L)	Measured Conc. (mg/L) ±SD
1	1.04 ±0.17
3	2.99 ±0.29
10	10.34 ±0.37
30	29.46 ±2.21
100	107.11 ±9.88

Inhibition of growth, fertility and reproduction of C.elegans

Exposure to NM100, no radiation

	Growth			Fertility			Reproduction		
Conc. TiO2 (mg/L)	Mean	SD	N	Mean	SD	N	Mean	SD	N
1	-3,01	1,44	4	0	0	4	-4,31	9,22	4
3	-3,14	1,38	4	0	0	4	2,58	5,41	4
10	-4,10	0,56	4	0	0	4	-4,64	6,11	4
30	-4,01	0,74	4	0	0	4	-3,64	11,17	4
100	-2,00	1,32	4	0	0	4	-8,95	7,72	4

Inhibition of growth, fertility and reproduction of C.elegans

Exposure to NM100 and simulated solar radiation (SSR)

SSR intensity: 231 W/m^2 (300-800 nm)

SSR duration: 30 min, 4 h after test start

	Growth inhibition [%]			Fertility inhibition [%]			Reproduction inhibition [%]		
Conc. TiO2 (mg/L)	Mean	SD	N	Mean	SD	N	Mean	SD	N
1	2,71	2,63	4	0	0	4	6,99	11,22	4
3	-0,30	1,86	4	0	0	4	0,43	23,82	4
10	-2,59	2,10	4	0	0	4	1,53	5,57	4
30	-0,30	1,54	4	0	0	4	1,00	11,36	4
100	-1,96	5,75	4	0	0	4	9,25	9,54	4

Attached document 3: Poster SETAC 2013_J Angelstorf.pdf (page 79): ENV/JM/MONO(2015)17/PART2/ANN2

Applicant's summary and conclusion

Conclusions

Study's author's conclusions: Reproduction of C.elegans is significantly inhibited by P25 at concentrations of 30 mg/L (LOEC 30 mg/L in reported test, 10 mg/L in 2 pre-tests). Simultaneous irradiation of C.elegans exposed to P25 increases the inhibition at the highest test concentration by a factor of approx. 2. The observed accumulation of TiO2 NPs in the intestinal tract of the worms might lead to starving of the worms.

6.3 Terrestrial toxicity

6.3.1 Toxicity to soil macroorganisms except arthropods

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

Administrative Data

Purpose flag key study ( ) robust study summary ( ) used for classification ( ) used for MSDS
Study result type experimental result
Study period November 2010 - June 2013
Reliability 1 (reliable without restriction)
Rationale for reliability in the style of GLP

Data source

Reference

Reference type study report
Author Anne Wyrwoll
Year
Title
Bibliographic source
Testing laboratory IBACON GmbH
Report no.
Owner company UBA, RWTH Aachen, IBACON GmbH
Company study no. Report date

Data access

data submitter is data owner

Materials and methods

Test guideline

Qualifier according to
Guideline OECD Guideline 207 (Earthworm, Acute Toxicity Tests)
Deviations no

GLP compliance

no in the style of

Test materials

Details on sampling
Soil samples were collected at the end of the experiment. Subsquently they were frozen at -20°C until analysis. Prior to analysis samples were dried in an oven for 15 h at 105°C. Dried samples were than ground finely with a ball mill.

Details on analytical methods
Ground soil samples were digested with a mixture of hydrofluoric acid, nitric acid and perchloric acid. Thereafter they were analyzed with inductive coupled plasma optical emission spectroscopy (ICP-OES, LOQ 5 ppb). Access to titanium analysis provided at the Natural History Museum London within the QualityNano scheme funded by the European Commission under FP7 Capacities Programme Grant Agreement No: 262163

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

Test organisms

Test organisms (species)
Eisenia fetida

Animal group
annelids

Study design

Study type
laboratory study
Test duration type
short-term toxicity
Substrate type
natural soil
Total exposure duration
14 d
Remarks
Post exposure observation period
1 day post exposure on wet paper to defecate

Test conditions

Test temperature
20°C
pH
5

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

Nominal and measured concentrations
Nominal: 1000 mg/kg
Measured: 878 mg/kg This is 88% of the nominal value.

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

Results and discussions

Effect concentrations

Duration	14 d
Endpoint	NOEC
Effect conc.	>= 1000 mg/kg soil dw
Nominal/Measured	nominal
Conc. based on	test mat.
Basis for effect	mortality
Remarks (e.g. 95% CL)	

Overall remarks, attachments

Attached document 4: Application of a Nanomaterial-Suspension to soil_12-04-30.pdf (page 80 - 84): ENV/JM/MONO(2015)17/PART2/ANN3

Attached document 2: SOP Preparation of a NM 100 suspension.pdf (page 76 - 78): ENV/JM/MONO(2015)17/PART2/ANN1

Applicant's summary and conclusion

Validity criteria fulfilled
yes

Conclusions
At a level of 1000 mg/kg NM 100 had no effect on the mortality of Eisenia fetida. The results of the titanium analysis of the test soils show that the application method described in the attached standard operating procedure resulted in a homogeneous and reproducible application of the TiO2 nanomaterial to the test soil.

Endpoint study record: Toxicity to soil macroorganisms except arthropods.002 by RWTH Aachen

Administrative Data

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

Data source

Reference
Reference type study report
Author Anne Wyrwoll **Year**
Title
Bibliographic source

Testing laboratory IBACON GmbH **Report no.**
Owner company UBA, RWTH Aachen, IBACON GmbH
Company study no. Report date
Data *access*
data submitter is data owner

Materials and methods

Test guideline

Qualifier according to
Guideline OECD Guideline 222 (Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei))
Deviations no

GLP compliance

no in hte style of GLP

Test organisms

Test organisms (species)
Eisenia fetida
Animal group
annelids

Study design

Study type
laboratory study
Test duration type
long-term toxicity
Substrate type
natural soil
Total exposure duration
56 d
Remarks

Test conditions

Test temperature
20°C
pH
Test initiation: 4.7-5.0
Test termination: 5.5-6.3
Moisture
55% of water holding capacity
Details on test conditions
Test soil: Natural reference soil RefeSol 01-A a natural, slightly loamy, middle acidic, very slightly humic soil was used as test soil. The soil was air dried, sieved through a 2 mm sieve and stored at room temperature until use.

Results and discussions

Effect concentrations

Duration	56 d
Endpoint	NOEC
Effect conc.	>= 1000 mg/kg soil dw

Nominal/Measured nominal

Conc. based on test mat.

Basis for effect reproduction

Remarks (e.g. 95% CL)

Overall remarks, attachments

Attached document 2: SOP Preparation of a NM 100 suspension.pdf (page 76 - 78): ENV/JM/MONO(2015)17/PART2/ANN1

Attached document 4: Application of a Nanomaterial-Suspension to soil_12-04-30.pdf (page 80 - 84): ENV/JM/MONO(2015)17/PART2/ANN3

Applicant's summary and conclusion

Conclusions

At a level of 1000 mg/kg NM 100 had no effect on the reproduction of Eisenia fetida.

7. TOXICOLOGICAL INFORMATION

7.1 Toxicokinetics, metabolism and distribution

7.1.1 Basic toxicokinetics

Endpoint study record: Basic toxicokinetics_NM 100_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

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 100

Test materials

Details on test material

Commercial name: Tiona AT-1 from Cristal Global

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 tissues: liver, spleen, kidneys, thymus, heart, lungs, lymph nodes (mesenteric and popliteal), brain, bone including bone marrow (femur), testes/ovaries, skin, muscle.Blood sampling: - single admin

(day 1): t=5, t=10, t=20, t=30 minutes,-Repeated admin (day 5): t=1, t=2, t=4, and t=8 hour

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
Attached document	D7 Acute Tox: **ENV/JM/MONO(2015)17/ANN11** D7__Biodistribution: **ENV/JM/MONO(2015)17/ANN112**
Remarks	Data in the report and detailes 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 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

http://www.nanogenotox.eu/ : **ENV/JM/MONO(2015)17/ANN22**

7.2 Acute Toxicity

7.2.1 Acute toxicity: oral

7.2.2 Acute toxicity: inhalation

Endpoint study record: Intratracheal instillation by Fraunhofer Institute of Toxicology & 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
Driscoll KE, Costa DL, Hatch G, Henderson R, Oberdorster G, Salem H, Schlesinger RB. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. Toxicol Sci. 2000 May; 55(1):24-35. Total dose given in two aliquots on two consecutive days, each suspended in 0.3 ml saline.

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

Attached document 5: OECD_TiO2_IT-Test_BAL_020513.pdf (page 85 - 92): ENV/JM/MONO(2015)17/ANN5

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

7.3 Irritation / corrosion

7.4 Sensitisation

7.5 Repeated dose toxicity

7.6 Genetic toxicity

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: buccal mucosa permeability by University of Graz

Administrative Data

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

Data source

Data access

other: performed and provided by University of Graz, Austria

Cross-reference to same study

Roblegg E., Fröhlich E., Samberger C., Zaversky M., Teubl B., Zimmer A., Evaluation of a physiological in-vitro system to study the transport of nanoparticles through the buccal mucosa, Nanotoxicology. 2011 May 18. [Epub ahead of print] PMID: 21591874 Teubl B., Meindl C., Eitzelmayer A., Zimmer A., Fröhlich E. and Roblegg E., In-vitro Permeability Studies of neutrally charged Polystyrene Particles through the Buccal Mucosa, submitted 2012 Teubl B., Leitinger G., Fröhlich E., Schneider M., Tockner M., Zimmer A., Roblegg E., Buccal Mucosa as a Route for TiO2 Uptake, in preparation

Materials and methods

Principles of method if other than guideline

Method/ guideline followed: Roblegg E., Fröhlich E., Samberger C., Zaversky M., Teubl B., Zimmer A., Evaluation of a physiological in-vitro system to study the transport of nanoparticles through the buccal mucosa, Nanotoxicology. 2011 May 18. [Epub ahead of print] PMID: 21591874 Teubl B., Meindl C.,

Eitzelmayer A., Zimmer A., Fröhlich E. and Roblegg E., In-vitro Permeability Studies of neutrally charged Polystyrene Particles through the Buccal Mucosa, submitted 2012

Describe the scientific and technical basis of the test method

What biological/cellular model is the method based on?

excised porcine buccal mucosa

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

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

(i.e., tetrazolinium reductase index (TR Index)). Additionally, the integrity of the tissue was checked. The integrity test of the membrane was carried out using methylene blue/ PBS (1mg/ml) and methylene blue/ EDTA/ PBS (1mg/0.5mM/ml).The oral barrier studies were performed with static Franz diffusion cells (PermeGear, USA, 11.28 mm jacketed cell with a flat ground (ground o-ring) joint and clear glass with an 8 ml receptor volume). Each cell consisted of a donor and of a receiver compartment. The receiver compartment was surrounded by a water jacket to assure a physiological temperature of 37 ± 0.5 °C throughout the experiment. The receiver compartment was filled with 7.8 ml PBS buffer and heated to 37 °C before use. A magnetic stirrer was used with an agitation of 300 rpm to assure equal distribution. Between the compartments the excised viable and integral sheet of mucosa was inserted and fixed with retainer clips in such a way that the epithelium faces the donor and the connective tissue region faces the receiver compartment. After an equilibration time of 30 min, the buffer in the donor compartment was replaced by TiO2 particles dispersed in PBS in a concentration of 100 µg/ml. After 4 h test duration, the mucosa was washed 3 times with PBS, fixed and embedded. Observation of the tissue samples was carried out by transmission electron microscopy and the particles were verified by element-analyses. • Analytics (analytical verification) The penetration behavior of NM100 particles was evaluated by Transmission Electron Microscopy (TEM). The tissue was fixed with 0.1M sodium phosphate buffered 2.5% glutaraldehyde overnight at 4°C and post-fixed in 1.0% osmium tetroxide. Dehydration was carried out through a graded series of ethanol to 100%. Subsequently, the tissue was transferred into propylene oxide and embedded into epoxy resin. Thin tissue sections were cut with a diamond knife and placed onto 300 mesh copper grids. The grids were not stained with heavy metals to prevent staining precipitates. Transmission Electron Microscopy images were obtained using a TEM model Tecnai equipped with an energy filter.

Performance assessment of the method

Test materials

Details on test material

in media: Zeta-Potential: -16.2 mV agglomeration size: mean diameter 705.6 nm (PdI 0.391) RB adsorption constant: 0.099 ml/mg

Sample preparation/conditioning protocol

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

Results and discussions

Remarks on results including tables and figures

The permeability of the NM100 across the buccal mucosa with a thickness of approximately 700 µm was determined. As illustrated in Figure 1,2 and 3 particles permeated the mucus layer and penetrated into the epithelium. It was found that particles were internalized by the cells of the superficial epithelium and

could also be found in the intercellular spaces. Additionally, NM100 particles were detected in the basal lamina, the deepest part of the buccal epithelium. The existence of all particles was evaluated/ confirmed by energy filtered TEM and verified by elemental mapping. The results of the permeability studies demonstrated that TiO2 particles (NM100) can permeate the mucus layer and penetrate into deep parts (basal lamina!) of the epithelium. Recent investigations showed that the buccal uptake is a function of the surface charge, the size and hydrophilicity/ hydrophobicity. It seems that in case of TiO2 NM 100 all 3 aspects (size: 148.25 nm, hydrophilicity: 0.099 ± 0.01 µg/ml RB binding constant and surface charge: nearly neutral) control buccal uptake. In previous studies it could be demonstrated that 200 nm positive and 200 nm neutral polystyrene nanoparticles were able to permeate the mucus layer readily and to penetrate the buccal epithelium to a high extent. 25 nm neutral particles also penetrated the mucosal tissue, however, the diffusion velocity was 172-fold lower and also uptake efficiency was reduced. It seems that the mucus layer together with the buccal epithelium acts as a stronger barrier for 25 nm particles than for 200 nm particles. Apart from the particle size, the buccal uptake behaviour is also influenced by the surface properties of nanoparticles. The degree of hydrophilicity affected the transport of polystyrene particles: 200 nm positive and 200 nm neutral particles showed appropriate hydrophilicity (0.04 ml/mg and 0.09 ml/mg) to permeate the mucus layer and, the buccal epithelium to a great extent.

Overall remarks, attachments

Attached background material

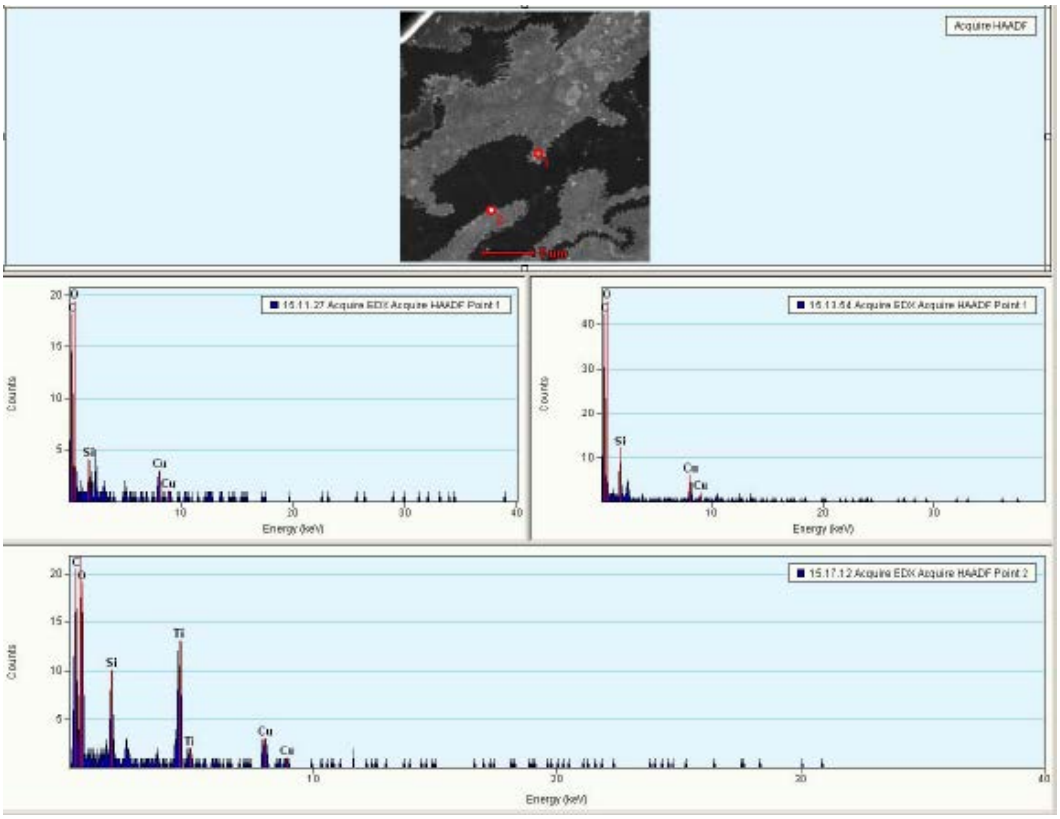


Figure 2 Electron microscopic images of TiO_2 particles NM 100 on the inside and on the outside of a buccal epithelial cell. Particles (red circles) are verified as Ti and O_2 via element analysis.

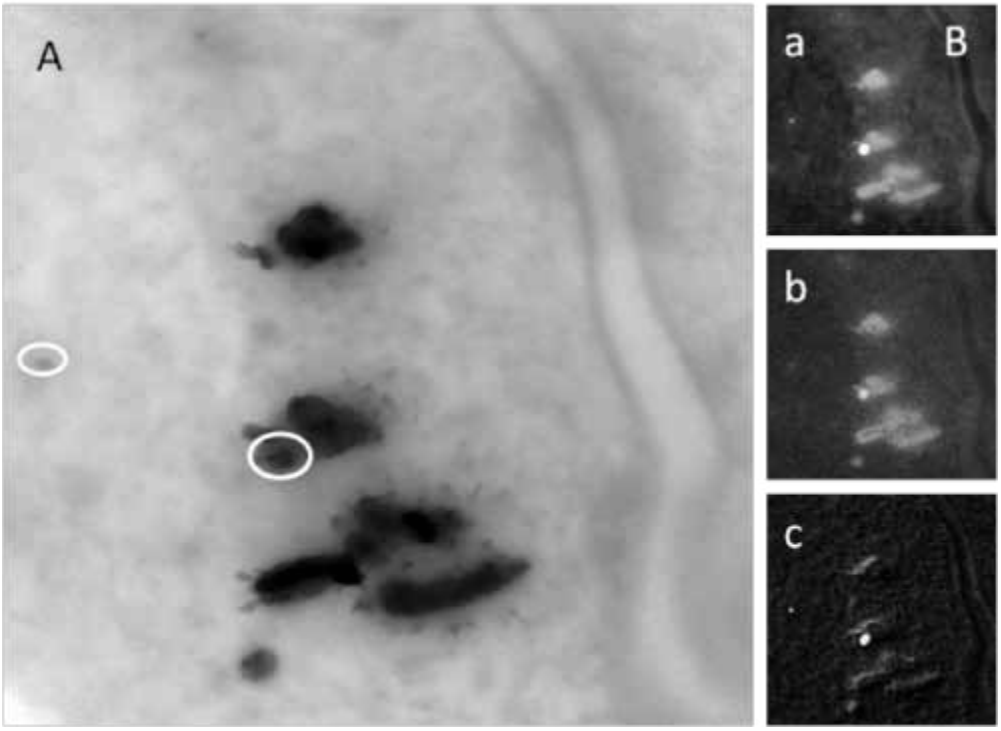


Figure 3 A) TEM image of TiO_2 particles NM 100 in the stratum superfaciale. B) The existence of TiO_2 was verified by elemental mapping. Images were acquired a) post-edge and b) pre-edge. c) The elemental Ti-mapping proves the existence of TiO_2.

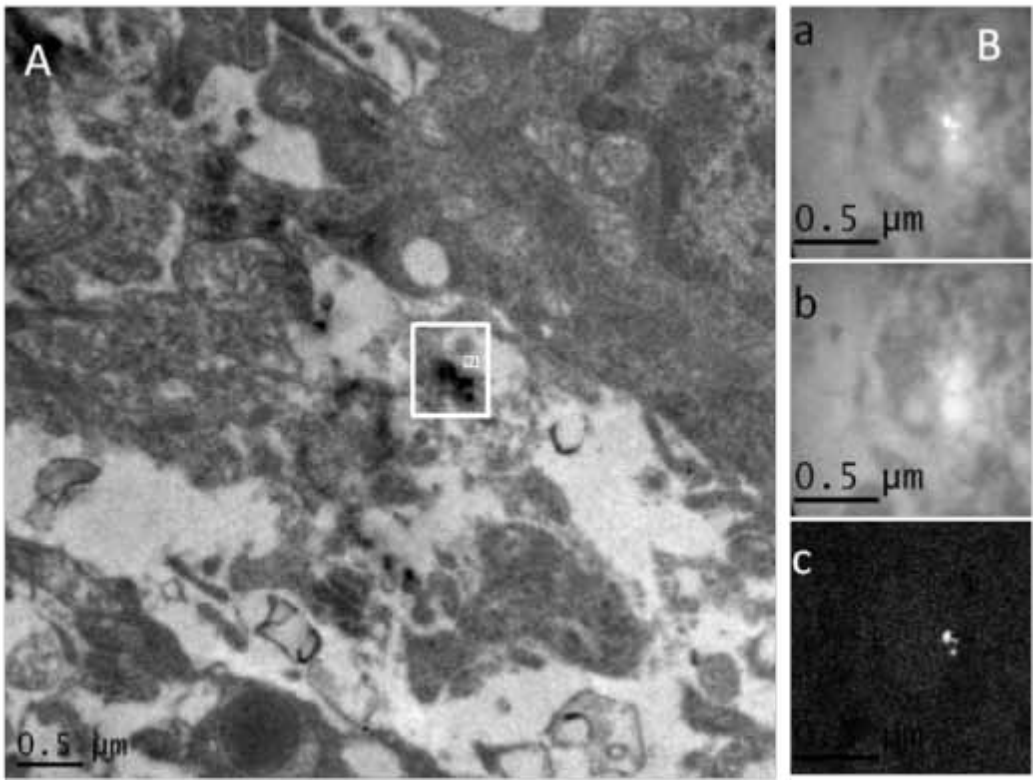


Figure 4 A) TEM image of TiO_2 particles NM 100 in the basal lamina. B) The existence of TiO_2 was verified by elemental mapping. Images were acquired a) post-edge and b) pre-edge. c) The elemental Ti-mapping proves the existence of TiO_2.

Applicant's summary and conclusion

Conclusions

The results of the permeability studies demonstrated that TiO2 particles can permeate the mucus layer and penetrate into deep parts (basal lamina!) of the epithelium.

Cross-reference to other study

R.H. Müller, in Colloidal Carriers for Controlled Drug Delivery and Targeting, CRC Press Stuttgart. 1991 p. 99-109.

Endpoint study record: cyto-toxicity by University of Graz

Administrative Data

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

Data source

Data access

other: performed and provided by University of Graz, Austria

Cross-reference to same study

Roblegg E., Fröhlich E., Samberger C., Zaversky M., Teubl B., Zimmer A., Evaluation of a physiological in-vitro system to study the transport of nanoparticles through the buccal mucosa, Nanotoxicology. 2011 May 18. [Epub ahead of print] PMID: 21591874 Teubl B., Meindl C., Eitzelmayer A., Zimmer A., Fröhlich E. and Roblegg E., In-vitro Permeability Studies of neutrally charged Polystyrene Particles through the Buccal Mucosa, submitted 2012 Teubl B., Leitinger G., Fröhlich E., Schneider M., Tockner M., Zimmer A., Roblegg E., Buccal Mucosa as a Route for TiO2 Uptake, in preparation

Materials and methods

Principles of method if other than guideline

Method/ guideline followed: Roblegg E., Fröhlich E., Samberger C., Zaversky M., Teubl B., Zimmer A., Evaluation of a physiological in-vitro system to study the transport of nanoparticles through the buccal mucosa, Nanotoxicology. 2011 May 18. [Epub ahead of print] PMID: 21591874 Teubl B., Meindl C., Eitzelmayer A., Zimmer A., Fröhlich E. and Roblegg E., In-vitro Permeability Studies of neutrally charged Polystyrene Particles through the Buccal Mucosa, submitted 2012

Describe the scientific and technical basis of the test method

What biological/cellular model is the method based on?

buccal squamous epithelial TR 146 cells

What biological endpoints/responses does this method address?

cytotoxicity

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

Formazan bioreduction: In order to examine cell viability, a CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used according to the instruction given by the manufacturer. 2 x 104 cells/200 µl medium were seeded in 96 well plates and cultured for 24 h. Subsequently, the medium was replaced by particles/serum-free medium dispersion in different concentrations and incubated for 4h and 24h. 20 µl of a MTS/PMS solution per well was added and re-suspended. After an incubation time of

4 h, the absorbance was measured at 490 nm with a VIS-plate reader (FLUOstar Optima, BMG, Labortechnik). LDH release: To evaluate the lactate dehydrogenase (LDH) release, 2 x 104 cells/200 µl medium were seeded in a 96 well plate and incubated for 24 h. The medium was replaced by a particles/serum-free medium and incubated for 4h and 24h. LDH leakage was determined using a CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer´s instruction. Control wells (100% LDH release) were treated with 2 µl of lysis solution. 25 µl of the supernatant were mixed with 25 µl of the CytoTox-ONE Reagent in a white microtiter plate. At the end of 10 min incubation time (at RT), reaction was stopped by adding 12.5 µl stop solution. The fluorescence was recorded by fluorometer (FLUOstar Optima, BMG, Labortechnik) at 560 nm excitation wavelength and 590 nm emission wavelength. Number of replica: n=6 Frequency of Dosing: - Positive and negative control groups and treatment: untreated cell as negative control Solvent: serum-free medium Description of follow up repeat study: same conditions Criteria for evaluating results: negative control, cell viability, seeding cell density (calculated from the gowth curve/prolifertation curve)

Performance assessment of the method

Test materials

Sample preparation/conditioning protocol

Particles dispersed in serum-free medium

Results and discussions

Remarks on results including tables and figures

The cytotoxic effects of NM100 were assessed by a MTS assay after 4 and 24h. The data obtained from the particles showed negligible reduced mitochondrial activity/viability, indicating no cytotoxic effects. After 4 and 24h incubation time, more than 90 % viability was maintained. The membrane integrity was assessed by LDH release. NM100 particles displayed no significant influence on the membrane integrity independent on the concentration within 24h. The results of the MTS- and LDH-tests are listed in table 1 and 2 and illustrated in figure 5 and 6.

Overall remarks, attachments

Overall remarks

Generally, nanoparticles are effective disrupters of cell plasma membranes (Lerouei et al.). There are two common types of disruption, i) nanoscale hole formation and ii) membrane thinning effects. A disruption of the membrane correlates with the enzyme leakage, dye diffusion, cytotoxicity and in-vitro particle uptake. Therefore, LDH and MTS investigations were performed. The results indicated that no membrane disruption occurred. These data could be confirmed by the viability assays. NM100 particles, tested in concentrations up to 200 µg/ml, do not affect the viability and the membrane integrity of human buccal epithel cells under in-vitro conditions (manuscript in preparation).

Attached background material

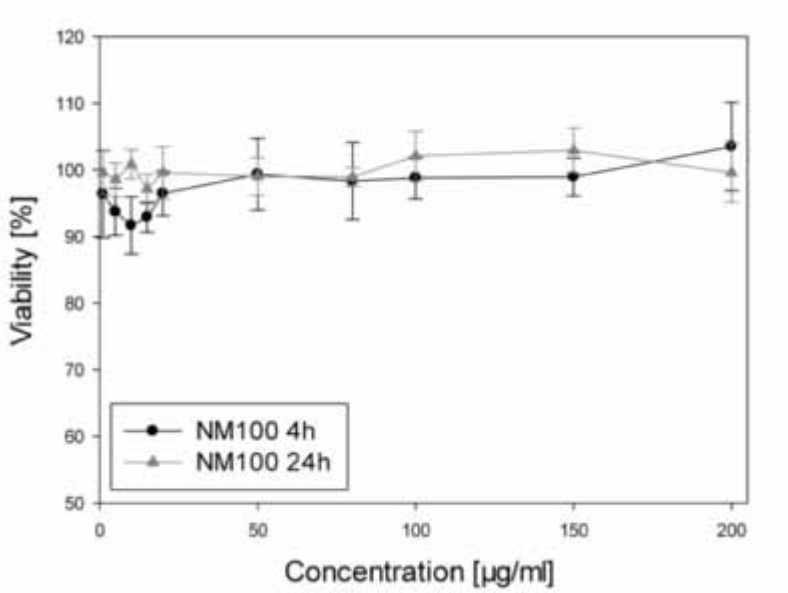


Figure 5 Viability assay (MTS-test) of TR 146 cells treated with NM100 particles (4h and 24 h incubation time).

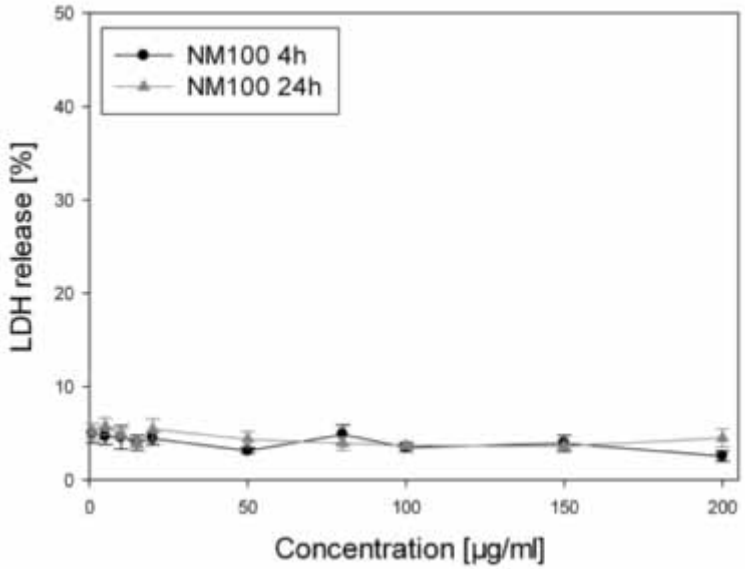


Figure 6 Membrane integrity assay (LDH-test) of TR 146 cells treated with NM100 particles (4h and 24 h incubation time).

Table 1 Results of the MTS-test of NM100

Concentration [µg/ml]	Viability [%] after 4 h	Standard deviation [%]	Viability [%] after 24 h	Standard deviation [%]
1	96,3732	6,5631	99,5941	3,2603
5	93,7195	3,5392	98,5642	2,5178
10	91,6862	4,3226	100,8502	2,1819
15	92,9103	2,2802	97,1667	2,2054
20	96,4737	3,3431	99,5961	3,8984
50	99,3452	5,4000	99,0253	2,8167
80	98,3371	5,7686	98,9505	1,3934
100	98,8027	3,1300	102,0807	3,6986
150	98,9416	2,8449	102,9377	3,3765
200	103,5028	6,6020	99,6028	4,4317

Table 2 Results of the LDH-test of NM100

Concentration [µg/ml]	LDH release [%] after 4 h	Standard deviation [%]	LDH release [%] after 24 h	Standard deviation [%]
1	4,9882	1,0393	5,1698	0,8692
5	4,6692	0,8913	5,7252	0,9076
10	4,5618	1,2507	4,9090	0,5243
15	3,9881	0,8169	3,9803	0,4850
20	4,4203	0,6550	5,4204	1,1365
50	3,1222	0,2710	4,3467	0,8217
80	4,8715	1,0054	3,9244	0,7351
100	3,4807	0,4483	3,6894	0,3780
150	3,9137	0,8516	3,6136	0,2916
200	2,5484	0,5252	4,4924	0,9765

Applicant's summary and conclusion

Cross-reference to other study

Leroueil P.R., Hong S., Mecke A., Baker J.R., Orr B.G., Banaszak Holl M.M., Acc. Chem. Res. 2007, 40, 335-342

8. ANALYTICAL METHODS

9. RESIDUES IN FOOD AND FEEDINGSTUFFS

10. EFFECTIVENESS AGAINST TARGET ORGANISMS

11. GUIDANCE ON SAFE USE