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DOSSIER ON ZINC OXIDE
- PART 2 -

Series on the Safety of Manufactured Nanomaterials
No. 52

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Series on the Safety of Manufactured Nanomaterials

No. 52

DOSSIER ON ZINC OXIDE
- PART 2 -

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. 53, *Dossier on Silver nanoparticles (2015)*

No. 54, *Dossier on Titanium dioxide (2015)*

ABOUT THE OECD

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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 format[6 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 the exploratory nature of the work would require subsequent follow-up work for example to review the

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

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

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

[7] SIAR = SIDS Initial Assessment Report (SIDS = Screening Information Data Set)

specific needs that may arise when performing risk assessment of nanomaterials. In this context, the programme's ultimate goal, to add to the knowledge of the properties of nanomaterials, would form a cornerstone.

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 Zinc Oxide. This nanomaterial 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. They have been analysed using OECD Guidelines for the Testing of Chemicals (TG)[8]. The data is presented in an IUCLID[9] style format and includes the protocols and methods used (see Preamble).

The Business & Industry Advisory Committee to the OECD (BIAC) via the Nanotechnology Industries Association (NIAC) led the Testing Programme on Zinc Oxide. This included the determination of the tests that were appropriate, performing a number of tests, as well as coordinating tests and results obtained by other the participating stakeholders. This programme has benefited from the co-sponsorship and the contribution of Australia, the U S Food and Drug Administration (FDA), and Spain.

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.

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

[9] IUCLID is a software programme 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.

ACKNOWLEDGMENTS

The OECD Secretariat and the Working Party on Manufactured Nanomaterials wish to thank the Business & Industry Advisory Committee to the OECD (BIAC) for leading the Testing Programme for Zinc Oxide. They are specifically grateful to David Carlander from the Nanotechnology Industries Association. In addition, we appreciate the efforts made by other countries that participated in the Testing Programme, in particular to Australia, the US Food and Drug Administration (FDA), and Spain.

TABLE OF CONTENTS

- Part 1 -

- Part 2 -

- Part 3 -

5. ENVIRONMENTAL FATE AND PATHWAYS

5.1 Stability

5.2 Biodegradation

5.3 Bioaccumulation

5.3.1 Bioaccumulation: aquatic / sediment

Endpoint study record: Disregarded.2010_06_29_Imperial College for PROSPECT_Isotopes_SSB

Administrative Data

Purpose flag	disregarded study		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	Only raw data are available.		

Data source

Data access

data submitter is data owner

Data protection claimed

yes, but willing to share

Materials and methods

Principles of method if other than guideline

Particles dissolved in 0.1M nitric acid.

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other: OECD Sponsorship Programme	NM110, NM111, NM112, NM113

Test material form

nanomaterial

Radiolabelling

no

Details on analytical methods

Multi-collector inductively coupled plasma mass spcetrometry.Standard-sample bracketing data correction.

Overall remarks, attachments

Remarks on results including tables and figures

Zn isotope composition falls within the range of natural values

Attached background material

Attached document	Remarks
NM_110_ANNEX_A72_091009ZnSSB.xls / 74.5 KB (application/octet-stream): **The attachments are available on the IUCLID 5 software or can be provided if requested**	
NM_110_ANNEX_A73_091014ZnSSB.xls / 91 KB (application/octet-stream): **The attachments are available on the IUCLID 5 software or can be provided if requested**	
NM_110_ANNEX_A74_091015ZnSSB.xls / 95 KB (application/octet-stream): **The attachments are available on the IUCLID 5 software or can be provided if requested**	

Applicant's summary and conclusion

Conclusions

NP is isotopically indistinguishable from natural materials & organisms.Isotopically labelled engineered nanoparticles required for tracing.

Endpoint study record: Disregarded.2010_06_29_Imperial College for PROSPECT_Isotopes_DS

Administrative Data

Purpose flag	disregarded study
Study result type	experimental result
Reliability	4 (not assignable)
Rationale for reliability incl. deficiencies	Only raw data are available.

Data source

Data access

data submitter is data owner

Data protection claimed

yes, but willing to share

Materials and methods

Principles of method if other than guideline

NPs dissolved in 0.1M nitric acid.

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other: OECD Sponsorship Programme	NM110, NM111, NM112, Nm113

Test material form

nanomaterial

Radiolabelling

no

Details on analytical methods

Double spike measurement technique employed.

Test conditions

Any other information on materials and methods incl. tables

For results files to operate correctly, keep contents of archived folders together in the given forma

Overall remarks, attachments

Attached full study report

Attached full study report
NM_110_ANNEX_A48_091102ZnDS.zip / 15.75 MB (application/octet-stream): **The attachments are available on the IUCLID 5 software or can be provided if requested**
NM_110_ANNEX_A49_091103ZnDS.zip / 11.77 MB (application/octet-stream): **The attachments are available on the IUCLID 5 software or can be provided if requested**
NM_110_ANNEX_A50_091116ZnDS.zip / 11.75 MB (application/octet-stream): **The attachments are available on the IUCLID 5 software or can be provided if requested**

Applicant's summary and conclusion

Conclusions

Double spike & standard sample bracketing technique yield the same result therefore either technique can be usedto achieve precise results.Isotopically labelled engineered nanoparticles required for tracing.

Endpoint study record: Disregarded.2010_11_04_ Imperial College for PROSPECT_Isotope_Tracing_Report

Administrative Data

Purpose flag	disregarded study		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	Insufficient for assessment. Only a method description is available.		

Data source

Data access

data submitter is data owner

Data protection claimed

yes, but willing to share

Materials and methods

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other: OECD Sponsorship Programme	NM110, NM111, NM112, NM113

Test material form

nanomaterial

Overall remarks, attachments

Attached full study report

Attached full study report
NM_110_ANNEX_A51_ZnO NM Tracing Imperia.pdf / 2.85 MB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**

5.4 Transport and distribution

Endpoint summary: Transport and distribution

Administrative Data

Discussion

Adsorption

The retention of uncoated non-nanoscale NM 110, coated nanoscale NM 111, and uncoated nanoscale NM 112 was examined in five soils with varying physical and chemical characteristics (CSIRO, 2012). The retention values (Kr) for all test items in soils were determined using the procedure by Cornelis et al. (2010). In addition, the solid-liquid partitioning (Kd) values for bulk ZnO (NM 113), soluble Zn, and geogenic Zn in soils were determined but only provided for NM 113 and soluble Zn. The Kd values of bulk ZnO (NM 113) and soluble Zn compared to the Kr values of NM 110, NM 111, and NM 112 were in the same order of magnitude. The highest Kr and Kd values for all test items were observed in the "Bute" soil.NM 110, NM 111 as well as NM 112 show a similar adsorption/desorption behaviour in different soils.

5.4.1 Adsorption / desorption

Endpoint study record: Key.2012-03-02_Australia_CSIRO_Retention

Administrative Data

Purpose flag	key study		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	No guideline followed, but sufficient for assessment.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
	Cornelis et al	2012	Retention of NM-110 in soils		Adelaide SA	00001	CSIRO		

Data access

data submitter is data owner

Data protection claimed

yes

Materials and methods

Study type

other: retention

Media

soil

Type of method

batch equilibrium method

Principles of method if other than guideline

Retention of NM-110 was examined in five soils with varying physical and chemical characteristics from Australia(see attached partitioning and retention methods).

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other: OECD Sponsorship programme	NM110, NM111, NM112, NM113

Study design

Batch equilibrium or other method

Any other information on materials and methods incl. tables

The retention values (Kr) for all test items in soils were determined using the procedure by Cornelis et al. (2010). In addition, the solid-liquid partitioning (Kd) values for bulk ZnO (NM 113), soluble Zn, and geogenic Zn in soils were determined but only provided for NM 113 and soluble Zn. The last one was used for the calculation of the Kr values. The Kr value is expressed as follow:

Kr = Msolid/MNP x L/S (Lkg-1)

MNP: small aggregates that pass 0.45 µm membrane filter
Msolid: manufactured NPs which aggregate or deposit on soil mineral that do not pass 0.45 µm membrane filter

Results and discussions

Results: Batch equilibrium or other method

Any other information on results incl. tables

See attached study reports for full data

Soils				NM-110
	Bulk ZnO	Soluble Zn	NM-110	0.45 µm-1kDa
	K_d(L kg^{-1})	K_d(L kg^{-1})	K_r(L kg^{-1})	%
Mt Compass	2.9 ± 0.06	2.3 ± 0.04	2.6 ± 0.27	2.9 ± 1.7
Ingham	2.3 ± 0.06	1.8 ± 0.04	> 4.5	bdl
Emerald Black	3.3 ± 0.04	2.8 ± 0.02	2.4 ± 0.03	3.8 ± 0.2
Bute	4.2 ± 0.04	> 5.6	> 4.5	bdl
Pt Kenny	3.8 ± 0.04	> 4.3	2.9 ± 0.22	1.4 ± 0.6

bdl=below detectable limits

Overall remarks, attachments

Attached background material

Attached document	Remarks
NM_110_ANNEX_A52_Cornelis et al.2010.pdf / 486.08 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN16**	

Attached full study report

Attached full study report
NM_110_ANNEX_A53_NanoHub_NM-110_Kd and Kr methods.docx / 70.15 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**
NM_110_ANNEX_A54_Kd and Kr value data_NM-110.docx / 12.41 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**
NM_111_ANNEX_A49_Kd and Kr value data_NM-111.docx / 12.91 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**
NM_111_ANNEX_A50_NanoHub_NM-111_Kd and Kr methods.docx / 73.66 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**
NM_112_ANNEX_A53_NanoHub_NM-112_Kd and Kr methods.docx / 70.06 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**
NM_112_ANNEX_A54_Kd and Kr value data_NM-112.docx / 12.41 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**

Applicant's summary and conclusion

Conclusions

NM-110, NM-111 as well as NM-112 show a similar adsorption/desorption behaviour in different soils.

Executive summary

The retention of uncoated non-nanoscale NM 110, coated nanoscale NM 111, and uncoated nanoscale NM 112 was examined in five soils with varying physical and chemical characteristics (CSIRO, 2012).

The retention values (Kr) for all test items in soils were determined using the procedure by Cornelis et al. (2010). In addition, the solid-liquid partitioning (Kd) values for bulk ZnO (NM 113), soluble Zn, and geogenic Zn in soils were determined but only provided for NM 113 and soluble Zn. The Kd values of bulk ZnO (NM 113) and soluble Zn compared to the Kr values of NM 110, NM 111, and NM 112 were in the same order of magnitude. The highest Kr and Kd values for all test items were observed in the "Bute" soil.NM 110, NM 111 as well as NM 112 show a similar adsorption/desorption behaviour in different soils.

6. ECOTOXICOLOGICAL INFORMATION

6.1 Aquatic toxicity

6.1.1 Short-term toxicity to fish

Endpoint summary: Short-term toxicity to fish

Administrative Data

Short description of key information

The 84-h EC50 values, determined on basis of hatching rate, were 2.065 mg/L for uncoated nanoscale ZnO and 2.066 mg/L for ZnO/bulk.

Discussion

In a non-GLP/guideline conform 96 h-embryo-larval bioassay according to Schulte & Nagel (1994), zebra fish (Danio rerio) embryos were exposed to non-OECD uncoated nanoscale ZnO (purity > 99%, particle size range: 50-360 nm) and the bulk counterpart ZnO/bulk (purity > 99%) at nominal concentrations of 0.1, 0.5, 1, 10 and 50 mg/L. Based on the mortality rate, the 96-h LC50 value of uncoated nanoscale ZnO and ZnO/bulk were 1.793 mg/L and 1.550 mg/L, respectively. The 84-h EC50 values, determined on basis of hatching rate, were 2.065 mg/L for uncoated nanoscale ZnO and 2.066 mg/L for ZnO/bulk.

Endpoint study record: Key.Nano.Zhu et al. (2008), Danio

Administrative Data

Purpose flag	key study; robust study summary		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	Acceptable, well documented publication/study report which meets basic scientific principles. Non-GLP conform study. Non-OECD NM was investigated. For Schulte & Nagel, 1994 a complete reference citation is missing.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Zhu, X. et al.	2008	Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (Danio rerio) early developmental stage	Journal of Environmental Science and Health Part A, 43: 278-284					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline available		

Principles of method if other than guideline

The zebrafish 96-h embryo-larval bioassay according to Schulte & Nagel (1994) was used to assess and compare the developmental toxicities of nanoscale zinc oxide (nZnO), titanium dioxide (nTiO2) and alumina (nAl2O3) aqueous suspensions. Toxicological endpoints such as zebrafish embryos or larvae survival, hatching rate and malformation were noted and described within 96 h of exposure. A comparative experiment with their bulk counterparts (i.e., ZnO/bulk, TiO2/bulk and Al2O3/bulk) was conducted to understand the effect of particle size on their toxicities.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	uncoated nanoscale ZnO
other: refrence item	ZnO/bulk

Details on test material

- Name of test material (as cited in study report): uncoated nanoscale ZnO
- Physical state: solid; nano-scale particles with a range of 50-360 nm

- Analytical purity: > 99%
Reference item:
- ZnO/bulk - purity: > 99.0%

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: nZnO ws added to 100 mL of Milli-Q® water and dispersed by ultrasonication at room temperature for 0.5 h
- Particle size: size was determined using a dynamic light scattering device (B19000AT, Brookhaven Instrument Corporation, USA) for particles of < 1μm. Determined sizes and morphology were confirmed using TEM, Tecnai G2T20ST, Philips, Holand); size range: 50 - 360nm

Test organisms

Test organisms (species)

Danio rerio

Details on test organisms

TEST ORGANISM
- Common name: Zebrafish
- Source: Market in Tianjin, China
- Age at study initiation (mean and range, SD): eggs
- Method of breeding: adult fish kept in 250 L full glass aquaria; 26 ± 1°C; 14h/10h light/dark cycle; roughly 2:1 male/female sex ratio; food: frozen chironomids larvae, twice a day
- Spawning: Spawning was triggered once the light was turned on and completed within 30 min; Eggs collected were rinsed several times with cleaning and aerated water to remove the residue on the egg surface; Normally fertilised eggs were picked out under a stereomicroscope for the experiments

Study design

Test type

static

Water media type

freshwater

Limit test

no

Total exposure duration

96 h

Test conditions

Nominal and measured concentrations

50, 10, 5, 1, 0.5, 0.1 mg/L and control (nominal)

Any other information on materials and methods incl. tables

The test was started as soon as the intact fertilized eggs were selected (within 1.5 hours postfertilization). Twenty-four eggs (blastula stage) were transferred into the test wells of a 24-well multi-plate so that each well contained one embryo. Actually. twenty wells were added 1 mL metal oxide particle suspension for each well, and the remaining four (Milli-Q water control) wells were prepared similarly, with 1 ml Milli-Q water replacing the particle suspensions. The concentration gradients were 50, 10, 5, 1, 0.5, 0.1 mg/L and 0 mg/L(Milli-Q®water control). To ensure a constant concentration, the wells of the 24-well multi-plate were covered with transparent plastic film, and slight shake was done at 12-h intervals throughout the 96-h of exposure time. All the 24-well multi-plates with experiment embryos were placed in an illumination incubator. During the test, the temperature was maintained at 26 ± 1°C with a 14-h/10-h,

light/dark cycle. Experiments were repeated 3 times.
The development status of zebrafish embryos and larvae were observed with an inverse microscope (x 10-40, DMLL, LeiKa Corp., Germany) and documented photographically at specified time points (t =6, 12, 24, 36, 48, 60, 72, 84, and 96-h), throughout the 96-h of exposure after fertilization. Endpoints used for assessing developmental toxicity included embryo-larvae survival and embryo's hatching rate. Malformations (e.g. pericardial edema and tissue ulceration,*etc.)*were also noted and described among the embryos and larva from both control and treated groups.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
96 h	LC50	1.793 mg/L	nominal	test mat.	mortality	95% CL, 1.498-2.145 mg/L
84 h	EC50	2.065 mg/L	nominal	test mat.	other: hatching rate	95% CL, 1.687-2.529

Details on results

Metal oxide particle-mediated malformation (e.g., pericardial edema and tissue ulceration, body arcuation, etc.) in the embryos and larvae from both the control and treatments was observed. When pericardial edema and body arcuation were considered, there was not a significant difference between the results of the nZnO suspensions treatment and the control. However, tissue ulceration of post-hatch zebrafish larvae exposed to nZnO suspension was observed to occur at 72 hpf. For the nZnO suspension at 5 mg/L particle concentration treatment, tissue ulceration was observed in zebrafish larve at 72 hpf, and its proportion to the surviving zebrafish was 31.67%. The percentage increased sharply to 95.83% and l00% at 96 hpf and 108 hpf respectively, which demonstrated that tissue ulceration became more evident as the exposure time increased and eventually resulted in some fish death. Compared to 5mg/L treatment, nZnO suspension with lower particle concentration caused a lower tissue ulceration rate in zebrafish larvae, and the time when ulceration occurred was delayed.

Any other information on results incl. tables

Table:Toxicological effects of ZnO suspensions at different particle concentration on survival and hatching zebrafish embryos and larvae at 96/84 hpf (all values are presented as mean± standard deviation, *p < 0.05, **p < 0.01 vs. control group)

	96 hpf survival (%)	84 hpf Hatching rate (%)
Particle Concentration		
Control (0 mg/L)	98.133± 3.233	98.333± 2.887
0.1 mg/L	96.467± 3.075	98.333± 2.887
0.5 mg/L	88.267±10.226	92.157± 6.585
1 mg/L	71.567± 9.241**	75.273± 3.199*
5 mg/L	28.133± 15.127**	29.803± 17.213**
10 mg/L	1.667± 2.887**	3.333± 5.774**
50 mg/L	0**	0**

Results and conclusions:

Of the substances tested ZnO was the most toxic material to zebrafish embryos and larvae, the 96-hLC50of nZnO and ZnO/bulk aqueous suspensions on the zebrafish survival were 1.793 mg/L and 1.550 mg/L, respectively; and the 84-h EC50 on the zebrafish embryo hatching rate were 2.065 mg/L and 2.066 mg/L, respectively. But none of the nTiOz, TiO2/bulk, nAl2O3 and Al2O3/bulk suspensions showed any significant toxicities to zebrafish embryos and larvae. The zebrafish developmental toxicity of either the nZnO or the ZnO/bulk suspensions hadaobvious dose-depending property. Metal oxide nanoparticles with

different chemical compositions have different zebrafish developmental toxicity. Stability of chemical compositionsofnanomaterials itself should be consideredasan important factor affecting their potential environmental impacts and biological effects.

Applicant's summary and conclusion

Conclusions

The 84-h EC50 values, determined on basis of hatching rate, were 2.065 mg/L for uncoated nanoscale ZnO and 2.066 mg/L for ZnO/bulk.

Executive summary

In a non-GLP/guideline conform 96 h-embryo-larval bioassay according to Schulte & Nagel (1994), zebra fish (Danio rerio) embryos were exposed to non-OECD uncoated nanoscale ZnO (purity > 99%, particle size range: 50-360 nm) and the bulk counterpart ZnO/bulk (purity > 99%) at nominal concentrations of 0.1, 0.5, 1, 10 and 50 mg/L. Based on the mortality rate, the 96-h LC50 value of uncoated nanoscale ZnO and ZnO/bulk were 1.793 mg/L and 1.550 mg/L, respectively. The 84-h EC50 values, determined on basis of hatching rate, were 2.065 mg/L for uncoated nanoscale ZnO and 2.066 mg/L for ZnO/bulk.

6.1.2 Long-term toxicity to fish

Endpoint summary: Long-term toxicity to fish

Administrative Data

Short description of key information

As no clear dose-dependent effect on growth or survival was observed with respect to exposure to ZnO nanoparticles in the range tested, both NOEC and LOEC values have been defined >= 540 µg/L.

Key value for chemical safety assessment

EC10/LC10 or NOEC for freshwater fish 540 µg/L

Discussion

In a GLP-conform study following OECD 201 (Fish, Early-life Stage Toxicity Test), Danio rerio embryos were exposed to NM-110 (purity > 99%) for 35 days at nominal concentrations of 7, 20, 60, 180 and 540 µg/L as well as to the reference materials, NM-113 and ionic zinc, at a nominal concentration of 180 µg/L. A slight delay in the hatching of larvae exposed to > 180 µg/L NM 110 was observed possible due to the result of a data bias from one replicate tank. Similar concentrations of NM 113 and ionic zinc (180 µg/L) indicated an inhibitory effect on growth compared to NM 110. Poor survival during the embryonic stage and immediately post hatching suggested that the batch of eggs used in this study were of poor quality. The post-hatch survival of control individuals was lower than the OECD recommended value of 75%. As no clear dose-dependent effect on growth or survival was observed with respect to exposure to ZnO nanoparticles in the range tested, both NOEC and LOEC values have been defined >= 540 µg/L.

Endpoint study record: Key.Sanders et al. (2012).Long-term toxicity to fish

Administrative Data

Purpose flag	key study		
Study result type	experimental result		
Reliability	1 (reliable without restriction)		

Rationale for reliability incl. deficiencies	GLP and guideline conform study. The post-hatch survival of control individuals was lower than the OECD 201 recommended value of 75%.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	M.B. Sanders, E.Roberts, B.P. Lyons and T.Hutchinson	2012	Effects of zinc oxide nanoparticles on survival and growth of Danio rerio		Cefas, Weymouth	C5198	University of Exeter		2012-01-05

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 210 (Fish, Early-Life Stage Toxicity Test)	

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	Z-COTE (NM-110)
other: reference material	microscaled ZnO (NM-113)

Details on test material

- Name of test material: Z-cote (NM-110)
- Molecular formula: ZnO
- Molecular weight: 81.41 g. mol-1
- Physical state: Solid
- Analytical purity: >99%
- Composition of test material, percentage of components:
- Lot/batch No.:248-254
- Other: Molecular weight of dissociated Zn 65.38 g.mol-1

Reference material:
- microscaled ZnO (NM-113)

Analytical monitoring

yes

Details on sampling

- Concentrations: Measurments of zinc concentrations in test solutions
- Sampling method: Test water acidified
- Sample storage conditions before analysis: 4 degree celsius

Details on analytical methods

IDENTIFICATION AND QUANTIFICATION OF TEST SUBSTANCE/PRODUCT
- Detection method (e.g. ECD, UV, MS, ICP-AES, ICP-MS): ICPMS

Vehicle

no

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: Protocol for nanoparticle dispersion developed by National Physics Laboratory. Briefly nanoparticles were made into a paste using a few drops of DI water and then 10 drops of water was added to the paste and slowly stirred with a glass rod. After this the remaing water is added and the solution is sonicated twice for 10 sec
- Controls: RO water
- Evidence of undissolved material (e.g. precipitate, surface film, etc): End of study a fine doposition of material in exposure tanks from 60-540µg/L ZnO NP. Much of the depositied material was either coating the natural biofilm in the tanks or was bound to dead Artemia and faeces, suggesting that much of the material added to the tank may have been rapidely depositied.

Test organisms

Test organisms (species)

Danio rerio

Details on test organisms

The test was initiated with Danio rerio(WIK strain) embryos from mass spawining stocks held at Cefas, Weymouth. Information on the orginal strain can be supplied by AstraZeneca where stocks originate from (8th Oct 2010). Fish were fed newly hatched Artemia spp. supplemented with commercial flake food daily. These fish supplied an F1 generation at Cefas from individual pairings. These F1 fish were then maintained in identical conditions to the parents prior to provision of embryos.

Study design

Test type

flow-through

Water media type

freshwater

Limit test

no

Total exposure duration

35 d

Post exposure observation period

Larval total length, larval dry weight, larval survival and delay in hatching

Test conditions

Hardness

General water hardness - 260+/- 20 mg/L
Carbonate Hardness (KH) - 190 +10 mg/L

Test temperature

25+/-2 °C

pH

8.107-8.192

Dissolved oxygen

Minimum 80% of the air saturated value

Salinity

N/A

Nominal and measured concentrations

540, 180, 60, 20 and 7 µg/L (NM-110)180 µg/L (NM-113)

Details on test conditions

TEST SYSTEM
- Test vessel: 10L glass aquaria modified with outflow at 8L capacity and 10L glass aquaria modified modified with 4 mid-volume capcity
- Type: open
- Material, size, headspace, fill volume: Glass, 10L, unknown and 8L
- Renewal rate of test solution (frequency/flow rate): wter inflow rate to mixing vessel - 200 ml/min and dosing inflow rate to mixing vessel - 2.5 ml/min
- No. of organisms per vessel: 30
- No. of vessels per concentration (replicates): 4
- No. of vessels per control (replicates): 4
- No. of vessels per vehicle control (replicates): N/A
- Biomass loading rate: N7A

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: Declorinated water from supply to the laboratory.
- Total organic carbon: <1 mg/L
- Particulate matter: <3 mg/L

OTHER TEST CONDITIONS
- Adjustment of pH:
- Photoperiod: 16 h light: 8 h dark
- Light intensity: 1500-1900 lux

TEST CONCENTRATIONS
- Range finding study
- Test concentrations: 540, 180, 60, 20 and 7 µg/L

Any other information on materials and methods incl. tables

Micro-scale ZnO reference substance and ZnCl2 reference substance are used to compare against nano-scale ZnO.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
32 d	NOEC	>= 540 µg/L	nominal	test mat.	other: larval total length	duration in days post hatching
32 d	NOEC	>= 540 µg/L	nominal	test mat.	other: larval dry weight	duration in days post hatching
32 d	NOEC	>= 540 µg/L	nominal	test mat.	other: larval survival	duration in days post hatching

					(mortality)	
7 d	NOEC	>= 60 µg/L	nominal	test mat.	other: delay in hatching	data based on cumulative number of larve hatching on d 4 and 5
32 d	LOEC	>= 32 µg/L	nominal	test mat.	other: larval total length	duration in days post hatching
32 d	LOEC	>= 540 µg/L	nominal	test mat.	other: larval dry weight	duration in days post hatching
32 d	LOEC	>= 540 µg/L	nominal	test mat.	other: larval survival	duration in days post hatching
7 d	LOEC	180 µg/L	nominal	test mat.	other: delay in hatching	data based on cumulative number of larve hatching on d 4 and 5

Details on results

- Observations on body length and weight: No dose-dependent effect on body-length or weight. 540µg/L had an effect on body length but not significant.
- Other biological observations: Post-hatch survival - no dose-dependent effect
- Mortality of control: <70% of post hatch survival of control individuals after 32 days
- Any observations (e.g. precipitation) that might cause a difference between measured and nominal values: No

Reported statistics and error estimates

Mean and stardard deviation (SD) were calculated using Excel 2007. Survival and development data were analysed using Stata/IC software v11.2 for Windows. REsponses of the form r/n were analysed as binomal responses within a generalized linear model (GLM), using number of eggs or number hatched as denominators. Length and weight were analysesd by anova and checked with Kruskal and Wallis anova of ranks when Bartlett´s test indicated uneqqual variance. When anove indicated some difference of means the pattern was examined using Scheffe post-hoc test. NOEC and LOEC values were derived from Wald tests on model parameters. Distributions of values and patterns of response were examined graphically.

Any other information on results incl. tables

Results:

Over the exposure period, post hatch survival of control individuals was <70% (at 32dph).

A summary of the observations on zebrafish hatching success, growth and survival of individuals exposed to ZnO nanoparticles (NM-110 = Z-COTE), ZnO bulk material (NM-113) and ionic zinc (Zn^{++})

Nominal test concentration (µg /L)	**Measured test concentration as mean ± SD(µg ZnO/L)**	**Larvae alive on day 35**	**Mean % survival at hatching and day 32 post hatch**		**Standard Length at 32d post hatch**	**Dry weight at 32d post hatch[3]**
			Hatching[1] (mean ± SD)	**32d ph[2] (mean ± SD)**	**(mm) (mean ± SD)**	**mg (mean ± SD)**
Control	N/A	38	62 (1.9)	62 (5.3)	17.4 (3.0)	13.9 (6.8)
Zn^{++}	N/A	30	58 (8.8)	44 (8.8)	14.5 (4.8)	9.9 (8.1)
NM-113 180	N/A	29	70 (15.6)	37 (11.5)	15.9 (4.6)	10.5 (8.1)
NM-110 540	N/A	28	65 (5.8)	83 (9.9)	17.3 (3.6)	11.2 (5.9)
NM-110 180	N/A	33	53 (2.7)	68 (11.1)	18.6 (3.3)	15.1 (7.1)
NM-110 60	N/A	29	64 (3.2)	38 (14.2)	17.2 (4.1)	12.3 (8.1)
NM-110 20	N/A	38	59 (7.4)	54 (19.8)	18.0 (2.8)	17.6 (8.2)
NM-110 7	N/A	56	57 (8.2)	43 (7.9)	16.2 (3.8)	13.5 (7.4)

[1]survival observations calculated from initial stocking numbers
[2]survival observations calculated from hatched individuals
[3]means based on censored data (Annex VII-Growth Data15.1)

Summary of life cycle effects in Zebrafish exposed to zinc oxide nanoparticles expressed as LOEC and NOEC concentrations based on nominal values.

End point	**DPH[a]**	**NOEC[b](µg/L)**	**LOEC[c](µg/L)**
Larval total length	32	≥540	>540
Larval dry weight	32	≥540	>540
Larval survival	32	≥540	>540
Delay in hatching	<7	≥60[d]	180[d]

[a]DPH = days post hatch
[b]NOEC = No-observed-effect concentration
[c]LOEC = Lowest –observed-effect concentration
[d]based on cumulative number of larvae hatching on day 4 and day 5

The results from the biological data are discussed for time to hatching, developmental and survival. There appears to be a slight delay in the hatching of larvae exposed to >180 µg/L nZnO, however, this may be the result of a data bias from one replicate tank and repetition of the study is required to confirm this finding. As no clear dose-dependent effect on growth or survival was observed with respect to exposure to ZnO nanoparticles in the range tested, both NOEC and LOEC values have been defined as > 540

µg/L. Data from the additional bulk ZnO and Zn^{++}positive control treatments suggest that at similar concentrations of 180 µg/L ionic zinc and bulk ZnO have an inhibitory effect on growth compared to nZnO. The poor growth rates, high mortality at the transition from yolk-sac stage to independent feeding and a notable number of undersized fish at the end of the study all indicate that the larvae were either not feeding properly or the dietary regime was sub-optimal. Following the transition to an Artemia only diet there was always prey items present in the water column of the exposure tanks and at termination on day 35, all individuals measured under the microscope were observed to have food in the gut.

Poor survival during the embryonic stage and immediately post hatching suggests that the batch of eggs used in this study were of poor quality. Additional handling and staging of individual eggs at the start of the study may also have attributed to the poor survival. Despite the low survival, no clear dose related effects following exposure to zinc oxide nanoparticles in the concentration range tested were observed were apparent.

Applicant's summary and conclusion

Conclusions

As no clear dose-dependent effect on growth or survival was observed with respect to exposure to ZnO nanoparticles in the range tested, both NOEC and LOEC values have been defined >= 540 µg/L.

Executive summary

In a GLP-conform study following OECD 201 (Fish, Early-life Stage Toxicity Test), Danio rerio embryos were exposed to NM-110 (purity > 99%) for 35 days at nominal concentrations of 7, 20, 60, 180 and 540 µg/L as well as to the reference materials, NM-113 and ionic zinc, at a nominal concentration of 180 µg/L. A slight delay in the hatching of larvae exposed to > 180 µg/L NM 110 was observed possible due to the result of a data bias from one replicate tank. Similar concentrations of NM 113 and ionic zinc (180 µg/L) indicated an inhibitory effect on growth compared to NM 110. Poor survival during the embryonic stage and immediately post hatching suggested that the batch of eggs used in this study were of poor quality. The post-hatch survival of control individuals was lower than the OECD recommended value of 75%. As no clear dose-dependent effect on growth or survival was observed with respect to exposure to ZnO nanoparticles in the range tested, both NOEC and LOEC values have been defined >= 540 µg/L.

6.1.3 Short-term toxicity to aquatic invertebrates

Endpoint summary: Short-term toxicity to aquatic invertebrates

Administrative Data

Discussion

Fabrega and Galloway (2010)
In a modified GLP-conform OECD 202 study, Daphnia magna was administrated with NM 112 and the reference materials NM 113 as well as ionic zinc for total exposure duration of 48 hours. Additionally, the feeding was assessed after 24 hours. The feeding rate of organisms exposed to 1 mg/L NM 112 was reduced up to 30% compared to the reference material. This effect was not related to a physical impairment or obstruction of the organism's feeding apparatus. Based on the mortality, the 48-h LC50 value of NM 112 and NM 113 were 1.55 mg/L and 3.32 mg/L, respectively.

Wiench et al. (2009)
Daphnia magna was exposed for 48 hours to nominal concentrations of 0.01 – 100 mg/L with NM 110 (purity > 99%), NM 111 (purity 96-99%), Z-COTE MAX (purity 96-99%) or non-nanoscale ZnO (< 1

µm, Sigma-Aldrich, corresponding to NM 113) according to OECD 202. The following 48 hour EC50 values (nominal) based on the mobility were determined: 7.5 mg/L (NM 110), 1.1 mg/L (NM 111), 1.0 mg/L (Z-COTE MAX) and 1.0 mg/L (non-nanoscale ZnO). Additionally, the EC50 value of NM 111 diluted in well-spring surface water and pond surface water was determined.

Heinlaan et al. (2008)

The toxicity of non-OECD NM (nano-sized ZnO (particle size: 50-70 nm), bulk ZnO and ZnSO4 · 7 H2O) was investigated to two crustaceans, Daphnia magna as well as Thamnocephalus platyrus under non-GLP/guideline conditions. No details on purity of the test items are indicated. The crustacea ecotox assays were performed according to the Standard Operational Procedures of Daphtoxkit F™ magna or Thamnoxkit F™, respectively. Using Daphnia magna, the following 48 h LC50 values were calculated: 3.2 mg/L (nano ZnO), 8.8 mg/L (bulk ZnO), and 6.1 mg/L (ZnSO4 · 7 H2O). Thamnocephalus platyrus was more sensitive to tested substances. The following 24 h LC50 values were estimated: 0.18 mg/L (nano ZnO), 0.24 mg/L (bulk ZnO), and 0.98 mg/L (ZnSO4 · 7 H2O).

Endpoint study record: WoE.Nano. Galloway (2010) Short-term toxicity to aquatic invertebrates

Administrative Data

Purpose flag	weight of evidence; robust study summary		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	The study was according to a modified OECD 202 guideline and GLP-conform. There is a discrepancy between the table title and the table header. In the table title, the ZnO bulk was mentioned, in the table header, ZnCl2 was written (see "any other information on results").		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Julia Fabrega and Tamara S. Galloway		Interim report for Ecotoxicology testing of manufacturing ZnO and CeO2 nanoparticles	http://www.nanotechia-prospect.org/managed_assets/files/ecotox_interim_report.pdf	University of Exeter and Birmingham		University of Exeter		2010-10-01

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
equivalent or similar to	other guideline: OECD 202	yes (additional endpoints)

Principles of method if other than guideline

Assessment of bioaccumulation of ZnO nanoparticles

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	Zinc oxide- Nanosun
other: reference item	ZnO bulk

Details on test material

- Name of test material (as cited in study report): Zinc oxide -Nanosun (NM-112)
- Physical state:solid
- Other: Particle size ~ 30 nm

Reference item:ZnO bulk (NM-113)

Analytical monitoring

no

Vehicle

no

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: Suspensions of ZnO NPs and bulk ZnO were prepared using standard operation protocols developed by the National Physics Laboratory (http://www.nanotechia-prospect.org). The NPs and bulk powders were made into a paste by adding one or two drops of deionized water (DIW) to 25 mg of particle mass and the mixture stirred with a metal spatula. Using a glass Pasteur pipet, 9−10 drops of DIW were then added slowly, continuing to mix the paste. The remaining DIW (250 mL, except for the few drops used to make the paste) was added to the paste and the suspension sonicated twice for 10 s using an ultrasonic probe (Cole Parmer 130 W Ultrasonic Processor). Bulk and ZnO NM suspensions were made freshly, immediately before dosing the exposure vessels.
- Controls: D.magna media
- Evidence of undissolved material (e.g. precipitate, surface film, etc):

Test organisms

Test organisms (species)

Daphnia magna

Details on test organisms

TEST ORGANISM
- Common name: D.Magna
- Strain:
- Source: University of Birmingham
- Feeding during test No
- Food type:
- Amount:
- Frequency:

ACCLIMATION
- Acclimation period:
- Acclimation conditions (same as test or not): same as test
- Type and amount of food: Cholorella vulgaris and Baker`s yeast
- Feeding frequency: daily
- Health during acclimation (any mortality observed):

QUARANTINE (wild caught)
- Duration:
- Health/mortality:

Study design

Test type

static

Limit test

no

Total exposure duration

48 h

Remarks and 24 h

Post exposure observation period

24 h after exposure feeding rate assessed

Test conditions

Test temperature

20°C

pH

7.5

Details on test conditions

TEST SYSTEM
- Test vessel: 250 ml beakers
- Type (delete if not applicable): open
- Material, size, headspace, fill volume: 100 ml of test media
- Aeration: Yes
- Type of flow-through (e.g. peristaltic or proportional diluter): N/A
- Renewal rate of test solution (frequency/flow rate):N/A
- No. of organisms per vessel: 30
- No. of vessels per concentration (replicates): 3
- No. of vessels per control (replicates):3
- No. of vessels per vehicle control (replicates): no vehicle used
- Biomass loading rate:

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water:
- Total organic carbon:
- Particulate matter:
- Metals:
- Pesticides:
- Chlorine:
- Alkalinity:
- Ca/mg ratio:
- Conductivity:
- Culture medium different from test medium:
- Intervals of water quality measurement:

OTHER TEST CONDITIONS
- Adjustment of pH:
- Photoperiod: 16 h light to 8 h dark

- Light intensity:
EFFECT PARAMETERS MEASURED (with observation intervals if applicable) :
TEST CONCENTRATIONS
- Spacing factor for test concentrations:
- Justification for using less concentrations than requested by guideline:
- Range finding study
- Test concentrations:
- Results used to determine the conditions for the definitive study:

Reference substance (positive control)

yes (Micro-size Zno and ionic ZnO used as reference material)

Any other information on materials and methods incl. tables

Micro-size Zno and ionic ZnO used as reference material to compare effects of ZnO NP. Bioimaging of ZnO and CeO2 uptake by D.magna by Coherent Anti stokes Raman Scattering and light microscopy.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
48 h	LC50	1.55 mg/L	nominal	other: NM-112	mortality	
48 h	LC50	3.32 mg/L	nominal	other: NM-113	mortality	

Results with reference substance

- Results with reference substance valid? Ionic Zn
- Mortality: yes 48 h acute testing
- LC50: 3.3 mg/L
- Other:

Reported statistics and error estimates

Anova

Any other information on results incl. tables

LC values for an acute (48h) exposure of ZnO MNP and bulk ZnO to 3-d old D. magna (EPA Probit analysis).

ZnCl2		95%confidence interval.		**ZnO MNP**		95% confidence interval.	
	Exposureconc(mgl-1)	lower	Upper		Exposureconc(mgl-1	lower	Upper
LC1	0.887	0.62	1.143	LC1	0.282	0.183	0.386
LC5	1.305	0.989	1.596	LC5	0.465	0.333	0.595
LC10	1.604	1.265	1.91	LC10	0.608	0.457	0.752
LC15	1.844	1.493	2.159	LC15	0.728	0.565	0.882
LC50	**3.321**	**2.932**	**3.713**	**LC50**	**1.557**	**1.335**	**1.796**
LC85	5.98	5.294	6.944	LC85	3.33	2.827	4.086
LC90	6.873	6.015	8.149	LC90	3.987	3.33	5.031
LC95	8.447	7.238	10.377	LC95	5.205	4.224	6.88
LC99	12.436	10.157	16.457	LC99	8.582	6.543	12.485

The feeding rate (uptake of the algae Chlorella vulgaris) was measured as a sublethal effect of exposure of D. Magna to ZnO MNP (Micronisers, APS 30 nm). Organisms exposed to ZnO MNP had a lower feeding rate (reduction of up to 30% at 1 mg l-1) compared to bulk ZnO and soluble zinc . Scanning electron micrographs suggested that the decrease in feeding rate was not related to a physical impairment or obstruction as a result of binding of ZnO MNP or ZnO MNP aggregates to the antenna nor thoracic appendages of the organisms.
Bioimaging using Coherent Anti-stokes Raman Scattering (CARS) and optical light microscopy confirmed that D. magna ingested large quantities of both MNPs, but in the case of CeO2 MNPs the high uptake did not affect survival.
See attached document section for graphs and images

Overall remarks, attachments

Attached background material

Attached document	Remarks
ecotox_interim_report 15.pdf / 158.69 KB (application/pdf): **ENV/JM/MONO(2015)15/ANN1**	
ecotox_interim_report 16.pdf / 323.35 KB (application/pdf): **ENV/JM/MONO(2015)15/ANN1**	

Applicant's summary and conclusion

Conclusions

Based on the mortality, the 48-h LC50 value of NM 112 and NM 113 were 1.55 mg/L and 3.32 mg/L, respectively.

Executive summary

In a modified GLP-conform OECD 202 study, Daphnia magna was administrated with NM 112 and the reference materials NM 113 as well as ionic zinc for total exposure duration of 48 hours. Additionally, the feeding was assessed after 24 hours. The feeding rate of organisms exposed to 1 mg/L NM 112 was reduced up to 30% compared to the reference material. This effect was not related to a physical impairment or obstruction of the organism’s feeding apparatus. Based on the mortality, the 48-h LC50 value of NM 112 and NM 113 were 1.55 mg/L and 3.32 mg/L, respectively.

Endpoint study record: WoE.Nano.Wiench et al. (2009), Daphnia

Administrative Data

Purpose flag	weight of evidence; robust study summary
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Guideline compliant. There is no data on GLP.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Wiench et al.	2009	Acute and chronic effects of nano- and non-nanocscaleTiO2 and ZnO particles on mobility and reproduction of the freshwater invertebrate Daphnia magna	Chemosphere 76 (10):1356-1365					

Materials and methods

Test guideline

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

Principles of method if other than guideline

Daphnia acute tests were performed according to OECD guideline 202. Besides nanoscale ZnO also non-nano scale ZnO was tested as well as nano- and non-nanoscale particles of TiO2 in comparison. Particles were either coated or uncoated and brought into suspension by ultrasonication or ultrasonication and stirring.

GLP compliance

no data

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	Z-COTE = NM-110
common name	Z-COTE HP1 = NM-111
common name	Z-COTE MAX
other: reference item	non-nanoscale ZnO

Details on test material

- Name of test material: zinc oxide as Z-Cote® (uncoated) = NM-110, as Z-Cote® HP1 (coated)= NM-111, and as Z-Cote® MAX (coated)
- Substance type: solid; uncoated or coated nano-scale particles
- Physical state: solid
- Analytical purity: > 99% (Z-Cote®), 96-99% (Z-Cote® HP1 and Z-Cote® MAX)

Reference item:
- non-nanoscale ZnO (< 1 µm, corresponding to NM-113)

Analytical monitoring

yes

Vehicle

no

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Z-Cote® and Z-Cote® MAX: A stock dispersion of 100 mg/L was prepared (medium: Artificial Elendt M4-medium). The dispersions was ultrasonicated for 5 minutes after transferring the test item into the test medium and stirred for 20 h.
- Z-Cote® HP1: Three stock dispersions of 100 mg/L were prepared (medium: Artificial Elendt M4-medium, pond surface water, or surface water of the well-spring of the River Selz in south-western Germany). The dispersions were ultrasonicated for 5 minutes.

Test organisms

Test organisms (species)

Daphnia magna

Details on test organisms

TEST ORGANISM
- Common name: water flea
- Source: laboratory stock culture at test facility
- Age at study initiation (mean and range, SD): third filial generation (F3) neonates, 2 - 24 h old- Method of breeding: reared in artificial fully defined M4 medium at 20 ± 2°C; medium was renewed twice weekly; food: unicellular green alga Desmodesmus subspicatus

Study design

Test type

static

Water media type

freshwater

Limit test

no

Total exposure duration

48 h

Test conditions

Hardness

2.02 - 2.86 mmol/L

Test temperature

20.0 - 21.0°C

pH

7.1 - 8.7

Dissolved oxygen

≥ 3 mg/L

Nominal and measured concentrations

5 concentrations: 0.01, 0.1, 1, 10, 100 (nominal) or 4 concentrations: 0.1, 1, 10, 100 (nominal) with 1 control

Details on test conditions

TEST SYSTEM
- Test vessel:
- Type: open
- Material, size, headspace, fill volume: glass tubes containing 10 mL test dispersion
- No. of organisms per vessel: 5
- No. of vessels per concentration (replicates): 4
- No. of vessels per control (replicates): 1

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: Artificial Elendt M4-medium according to OECD guideline 202
- Conductivity: 577 - 605 µS/cm

OTHER TEST CONDITIONS
- Photoperiod: 16 h

EFFECT PARAMETERS MEASURED (with observation intervals if applicable) : Immobilization at 24 and 48 h

TEST CONCENTRATIONS
- Spacing factor for test concentrations: 10
- Justification for using less concentrations than requested by guideline:
- Range finding study
- Test concentrations: 0.01, 0.1, 1, 10, 100 mg/L

Reference substance (positive control)

yes (potassium dichromate)

Any other information on materials and methods incl. tables

Analysis of particle size distribution:

The actual aggregate size distribution was assessed by analytical ultracentrifugation. At an acceleration of up to 300,000 g, solutes and nanoparticles sediment into fractions that are separated according to their size in the range of 0.5 to 10,000 nm. Agglomerates larger than ~10 µm diameter sediment before data acquisition starts and cannot be quantified. Simultaneous detection by synchronized optics quantifies the amount and the diameter of each fraction independently (Cölfen, 2004). A Beckman model XL ultracentrifuge that was modified for the online recording of sedimentation with turbidity, interference, and Schlieren or ultraviolet (UV) detection (Mächtle and Börger, 2006) was used. The evaluation of the ultracentrifugation raw data incorporates the fractal morphology of nanoparticle aggregates and applies the fractional dimension of 2.1 together with the sedimentation relation as specified by Lin et al. (1990). This value of the fractional dimension has been shown to be universal for all reaction-limited colloid aggregates Lin et al. (1990). If the fractional dimension of the aggregates were higher (lower) in the specific test substance application preparation, corresponding to a more compact (loose) structure of the aggregates, the retrieved particles sizes would shift to lower (higher) values.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
48 h	EC10	5.2 mg/L	nominal	test mat.	mobility	Z-Cote ; M4-medium
48 h	EC50	7.5 mg/L	nominal	test mat.	mobility	Z-Cote; M4-medium
48 h	EC10	0.2 mg/L	nominal	test mat.	mobility	Z-Cote HP1; M4-medium
48 h	EC50	1.1 mg/L	nominal	test mat.	mobility	Z-Cote HP1; M4-medium
48 h	EC10	2.7 mg/L	nominal	test mat.	mobility	Z-Cote HP1; medium: well-spring surface water
48 h	EC50	> 100 mg/L	nominal	test mat.	mobility	Z-Cote HP1; medium: well-spring surface water
48 h	EC10	9.3 mg/L	nominal	test mat.	mobility	Z-Cote HP1; medium: pond surface water
48 h	EC50	13.4 mg/L	nominal	test mat.	mobility	Z-Cote HP1; medium: pond surface water
48 h	EC10	0.7 mg/L	nominal	test mat.	mobility	Z-Cote MAX; M4-medium
48 h	EC50	1 mg/L	nominal	test mat.	mobility	Z-Cote MAX; M4-medium
48 h	EC10	0.6 mg/L	nominal	test mat.		non-nanoscale ZnO particles, M4 - medium
48 h	EC50	1 mg/L	nominal	test mat.		non-nanoscale ZnO particles, M4 - medium

Details on results

- Mortality of control: well below 10%

Results with reference substance

- Results with reference substance valid? yes
- EC50: experiments with reference substance regularly at intervals of 1-2 months, whereby the median effective concentration (EC50) after 24 h ranged from 0.6 to 1.4 mg/L during the last 10 months

Any other information on results incl. tables

Results and conclusion:
In all acute toxicity tests the survival in the controls was well above 90%. Hence, the validity criterion as required in the OECD guideline 202 was met.
Toxic effects on Daphnia magna - with EC50 values between 1 and 10 mg l^{-1}using M4 medium - were observed in six acute toxicity tests performed with ZnO-based pigments. Differences in effects on daphnids could be found neither using non-nano-scale or nano-scale nor coated or non-coated ZnO pigments. The use of natural water (both PW and SW) resulted in a decrease of acute toxicity concerning Z-COTE®HP1.
The acute effects of ZnO on the mobility ofDaphnia magnaare probably due to the ion toxicity of Zn and not ZnO.

Applicant's summary and conclusion

Conclusions

The following 48 hour EC50 values (nominal) based on the mobility were determined: 7.5 mg/L (NM 110), 1.1 mg/L (NM 111), 1.0 mg/L (Z-COTE MAX) and 1.0 mg/L (non-nanoscale ZnO).

Executive summary

Daphnia magna was exposed for 48 hours to nominal concentrations of 0.01 – 100 mg/l with NM 110 (purity > 99%), NM 111 (purity 96-99%), Z-COTE MAX (purity 96-99%) or non-nanoscale ZnO (< 1 µm, Sigma-Aldrich, corresponding to NM 113) according to OECD 202. The following 48 hour EC50 values (nominal) based on the mobility were determined: 7.5 mg/L (NM 110), 1.1 mg/L (NM 111), 1.0 mg/L (Z-COTE MAX) and 1.0 mg/L (non-nanoscale ZnO). Additionally, the EC50 value of NM 111 diluted in well-spring surface water and pond surface water was determined.

Endpoint study record: WoE.Nano.Heinlaan et al. (2008), Daphnia

Administrative Data

Purpose flag	weight of evidence		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	Acceptable, well documented publication/study report which meets basic scientific principles. Non-GLP/guideline conform study.Non-OECD NM was investigated.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Heinlaan, M. et al.	2008	Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus	Chemosphere 71: 1308-1316					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Crustacea ecotox assay was performed according to the Standard Operational Procedures of Daphtoxkit F™ magna (1996). Besides solutions with nanoparticles of ZnO also nanoparticles of TiO2 and CuO are tested in comparison as well as bulk oxides of the three metals and ZnSO4•7H2O. Additional tests with metal-specific biosensors are conducted in order to differentiate between toxic effects of metal oxides per se and solubilised metal ions.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	nano-sized ZnO
other: reference item	ZnO bulk
other: reference item	ZnSO4 heptahydrate

Details on test material

- Name of test material: nano-sized ZnO
- Physical state: solid
- Analytical purity: no data
- Storage condition of test material: stock suspensions stored in the dark at 4°C

Reference item:- ZnO bulk and ZnSO4 heptahydrate

Details on analytical methods

The bioavailable metal ions were quantified usingrecombinant metal sensor bacteria in which bioluminescenceis induced by intracellular metal ions. The inductionis mediated by a specific protein that recognizes therespective metal ion and regulates a promoter controllingthe expression of luxCDABE genes leading to bioluminescence.

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: Stock suspensions in Milli-Q (40g/L) were sonicated for 30 min.

Test organisms

Test organisms (species)

Daphnia magna

Details on test organisms

TEST ORGANISM
- Common name: water flea (Crustacea), neonates

Study design

Test type

static

Water media type

freshwater

Limit test

no

Total exposure duration

48 h

Test conditions

Test temperature

20°C

pH

7.3 - 7.8

Details on test conditions

TEST SYSTEM
- No. of vessels per concentration (replicates): 4 replicates were done.
TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: Test compound was diluted in synthetic freshwater (diluent included in the test kit, also used as a control).

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
48 h	LC50	3.2 mg/L	nominal	test mat.	mortality	
48 h	LC50	2.6 mg/L	nominal	element (Zn)	mortality	
48 h	other: LC20	2.45 mg/L	nominal	test mat.	mortality	
48 h	other: LC20	2 mg/L	nominal	element (Zn)	mortality	
48 h	NOEC	0.5 mg/L	nominal	test mat.	mortality	

Any other information on results incl. tables

All Zn formulations were very toxic: L(E)C50 (mg/L) for bulk ZnO, nanoZnO and ZnSO4 •7H2O: 8.8, 3.2, 6.1. The toxicity was due to solubilized Zn ions as proved with recombinant Zn-sensor bacteria.

Applicant's summary and conclusion

Conclusions

The following 48 h LC50 values were calculated: 3.2 mg/L (nano ZnO), 8.8 mg/L (bulk ZnO), and 6.1 mg/L (ZnSO4 · 7 H2O).

Executive summary

The toxicity of non-OECD NM (nano-sized ZnO (particle size: 50-70 nm), bulk ZnO and ZnSO4 · 7 H2O) was investigated in Daphnia magna under non-GLP/guideline conditions. The assay was performed according to the Standard Operational Procedures of Daphtoxkit F™ magna. The following 48 h LC50 values were calculated: 3.2 mg/L (nano ZnO), 8.8 mg/L (bulk ZnO), and 6.1 mg/L (ZnSO4 · 7 H2O).

Endpoint study record: WoE.Nano.Heinlaan et al. (2008), Thamnocephalus

Administrative Data

Purpose flag	weight of evidence		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	Non-GLP/guideline conform study. Acceptable, well documented publication/study report which meets basic scientific principles. Non-OECD NM was investigated.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Heinlaan, M. et al.	2008	Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus	Chemosphere 71: 1308-1316					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Crustacea ecotox assay was performed according to the Standard Operational Procedures of Thamnotoxkit F™ (1995). Besides solutions with nanoparticles of ZnO also nanoparticles of TiO2 and CuO are tested in comparison as well as bulk oxides of the three metals and ZnSO4•7H2O. Additional tests with metal-specific biosensors are conducted in order to differentiate between toxic effects of metal oxides per se and solubilised metal ions.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	nano-sized ZnO
other: reference item	ZnO/bulk
other: reference item	ZnSO4 heptahydrate

Details on test material

- Name of test material: nano-sized ZnO
- Physical state: solid
- Analytical purity: no data

Reference item:

- ZnO/bulk and ZnSO4 heptahydrate
- Storage condition of test material: stock suspensions stored in the dark at 4°C

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: Stock suspensions in Milli-Q (40g/L) were sonicated for 30 min.

Test organisms

Test organisms (species)

other: Thamnocephalus platyurus

Details on test organisms

TEST ORGANISM
- Common name: fairy shrimp (Crustacea)

Study design

Test type

static

Water media type

freshwater

Limit test

no

Total exposure duration

24 h

Test conditions

Test temperature

25°C

pH

7.3 - 7.8

Details on test conditions

TEST SYSTEM
- No. of vessels per concentration (replicates): 3 replicates were done.

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: Test compound was diluted in synthetic freshwater (diluent included in the test kit, also used as a control).

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
24 h	LC50	0.18 mg/L	nominal	test mat.	mortality	
24 h	LC50	0.14 mg/L	nominal	element (Zn)	mortality	
24 h	other: LC20	0.12 mg/L	nominal	test mat.	mortality	
24 h	other: LC20	0.09 mg/L	nominal	element (Zn)	mortality	
24 h	NOEC	0.03	nominal	test mat.	mortality	

Any other information on results incl. tables

All Zn formulations were very toxic: L(E)C50 (mg/L) for bulk ZnO, nanoZnO and ZnSO4•7H2O: 0.24, 0.18, 0.98. The toxicity was due to solubilized Zn ions as proved with recombinant Zn-sensor bacteria.

Applicant's summary and conclusion

Conclusions

The following 24 h LC50 values were estimated: 0.18 mg/L (nano ZnO), 0.24 mg/L (bulk ZnO), and 0.98 mg/L (ZnSO4 · 7 H2O)

Executive summary

The toxicity of non-OECD NM (nano-sized ZnO (particle size: 50-70 nm), bulk ZnO and ZnSO4 · 7 H2O) was investigated in Thamnocephalus platyrus under non-GLP/guideline conditions. The assay was performed according to theStandard Operational Procedures of Thamnoxkit F™. The following 24 h LC50 values were estimated: 0.18 mg/L (nano ZnO), 0.24 mg/L (bulk ZnO), and 0.98 mg/L (ZnSO4 · 7 H2O).

Endpoint study record: Disregarded.Nano.RA ZnSO4. Short-term toxicity to aquatic invertebrates.Blinova et al 2010. sp3. 024

Administrative Data

Purpose flag	disregarded study		
Study result type	read-across based on grouping of substances (category approach)		
Reliability	3 (not reliable)		
Rationale for reliability incl. deficiencies	ZnSO4 is not relevant for assessment.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Blinova I, Ivask A Heinlaan M, Mortimer M and Kahru A.	2010	Ecotoxicity of nanoparticles of CuO and ZnO in natural water.	Environmental pollution 158, 41-47					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline available		

Principles of method if other than guideline

test according to standardised "Protoxkit F"

GLP compliance

no data

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

no

Test material identity

Identifier	Identity
CAS name	zinc sulphate
CAS number	7733-02-0

Analytical monitoring

yes

Details on analytical methods

zinc measurements according to ISO 17294-2:2003

Details on test solutions

tests were done in artificial water and 6 natural river waters.

Test organisms

Test organisms (species)

other aquatic crustacea: Tetrahymena thermophila

Details on test organisms

Protoxkit F, 2 replicates

Study design

Test type

static

Water media type

freshwater

Total exposure duration

24 h

Test conditions

Hardness

Ca++ in natural waters varied between 124 and 58 mg/l; artificial medium CaCl2.2H20: 294mg/l

Test temperature

30°C

pH

pH of natural waters varied between 7.5 and 8.2, artificial water: 7.8

Nominal and measured concentrations

Zn background of natural waters baried between 1.4 and 3.1 mg (total)/l

Details on test conditions

The substance was added to the food substrate suspension in the test medium. While a normal proliferating protozoan culture clears the substrate suspension in 24hrs, inhibition of growth is reflected by residual turbidity measured by optical density at 440nm.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
24 h	EC50	7.1 mg/L	meas. (not specified)	dissolved	other: growth inhibition	artificial water
24 h	EC50	21.1 mg/L	meas. (not specified)	dissolved	other: growth inhibition	river 2
24 h	EC50	18.6 mg/L	meas. (not specified)	dissolved	other: growth inhibition	river 4
24 h	EC50	> 22 mg/L	meas. (not specified)	dissolved	other: growth inhibition	river 5

Applicant's summary and conclusion

Validity criteria fulfilled

yes

Conclusions

Tests done on standardised toxkit.Good quality and considered useful for assessing acute aquatic ecotoxicity.

Executive summary

Standardised toxkit test on Tetrahymena thermophila to check the effect of ZnO nanoparticles. This study record contains the results on ZnSO4 control (see results table).

Endpoint study record: Disregarded.Nano.RA ZnSO4. Short-term toxicity to aquatic invertebrates.Blinova et al 2010. sp2. 024

Administrative Data

Purpose flag	disregarded study		
Study result type	read-across based on grouping of substances (category approach)		
Reliability	3 (not reliable)		
Rationale for reliability incl. deficiencies	ZnSO4 is not relevant for assessment.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Blinova I, Ivask A Heinlaan M, Mortimer M and Kahru A.	2010	Ecotoxicity of nanoparticles of CuO and ZnO in natural water.	Environmental pollution 158, 41-47					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline available		

Principles of method if other than guideline

test done with "Thamnotoxkit F"

GLP compliance

no data

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

no

Test material identity

Identifier	Identity
CAS name	zinc sulphate
CAS number	7733-02-0

Details on test material

ZnSO4.7H20 from Sigma-Aldrich

Analytical monitoring

yes

Details on analytical methods

zinc measurements according to ISO 17294-2:2003

Details on test solutions

tests were done in artificial water and 6 natural river waters.

Test organisms

Test organisms (species)

other aquatic crustacea: Thamnocephalus platyurus

Details on test organisms

origin: Thamnotoxkit T

Study design

Test type

static

Water media type

freshwater

Total exposure duration

24 h

Test conditions

Hardness

Ca++ in natural waters varied between 124 and 58 mg/l; artificial medium CaCl2.2H20: 294mg/l

Test temperature

25°C

pH

pH of natural waters varied between 7.5 and 8.2, artificial water: 7.8

Nominal and measured concentrations

Zn background of natural waters baried between 1.4 and 3.1 mg (total)/l

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
24 h	EC50	0.22 mg/L	meas. (not specified)	dissolved	mortality	artificial water
24 h	EC50	0.92 mg/L	meas. (not specified)	dissolved	mortality	river 1
24 h	EC50	1.6 mg/L	meas. (not specified)	dissolved	mortality	river 2
24 h	EC50	0.61 mg/L	meas. (not specified)	dissolved	mortality	river 3
24 h	EC50	0.75 mg/L	meas. (not specified)	dissolved	mortality	river 4
24 h	EC50	1.1 mg/L	meas. (not specified)	dissolved	mortality	river 5
24 h	EC50	1.7 mg/L	meas. (not specified)	dissolved	mortality	river 6

Applicant's summary and conclusion

Validity criteria fulfilled

yes

Conclusions

Tests done according to standard protocol. Good quality and considered useful for assessing acute aquatic ecotoxicity.

Executive summary

Standardised thamnotoxkit test on Thanocephalus platyurus to check the effect of ZnO nanoparticles. This study record contains the results on the ZnSO4 control (see results table).

Endpoint study record: Disregarded.Nano.RA ZnSO4. Short-term toxicity to aquatic invertebrates.Blinova et al 2010. sp1. 024

Administrative Data

Purpose flag	disregarded study		
Study result type	read-across based on grouping of substances (category approach)		
Reliability	3 (not reliable)		
Rationale for reliability incl. deficiencies	ZnSO4 is not relevant for assessment.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Blinova I, Ivask A Heinlaan M, Mortimer M and Kahru A.	2010	Ecotoxicity of nanoparticles of CuO and ZnO in natural water.	Environmental pollution 158, 41-47					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 202 (Daphnia sp. Acute Immobilisation Test)	yes (Daphtoxkit T)

GLP compliance

no data

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

no

Test material identity

Identifier	Identity
CAS name	zinc sulphate
CAS number	7733-02-0

Details on test material

ZnSO4.7H20 from Sigma-Aldrich

Analytical monitoring

yes

Details on analytical methods

zinc measurements according to ISO 17294-2:2003

Details on test solutions

tests were done in artificial water and 6 natural river waters.

Test organisms

Test organisms (species)

Daphnia magna

Details on test organisms

origin: Daphtoxkit T

Study design

Test type

static

Water media type

freshwater

Total exposure duration

48 h

Test conditions

Hardness

Ca++ in natural waters varied between 124 and 58 mg/l; artificial medium CaCl2.2H20: 294mg/l

Test temperature

20°C

pH

pH of natural waters varied between 7.5 and 8.2, artificial water: 7.8

Nominal and measured concentrations

Zn background of natural waters baried between 1.4 and 3.1 mg (total)/l

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
48 h	EC50	1.4 mg/L	meas. (not specified)	dissolved	mobility	artificial water
48 h	EC50	1.8 mg/L	meas. (not specified)	dissolved	mobility	river 1
48 h	EC50	2 mg/L	meas. (not specified)	dissolved	mobility	river 2
48 h	EC50	1.6 mg/L	meas. (not specified)	dissolved	mobility	river 3
48 h	EC50	2.5 mg/L	meas. (not specified)	dissolved	mobility	river 4
48 h	EC50	1.4 mg/L	meas. (not specified)	dissolved	mobility	river 5
48 h	EC50	1.7 mg/L	meas. (not specified)	dissolved	mobility	river 6

Applicant's summary and conclusion

Validity criteria fulfilled

yes

Conclusions

Tests done according to standard protocol. Good quality and considered useful for assessing acute aquatic ecotoxicity.

Executive summary

Standard Daphnia magna testing to check the effect of ZnO nanoparticles. This study record contains the results on the ZnSO4 control (see results table).

6.1.5 Toxicity to aquatic algae and cyanobacteria

Endpoint summary: Toxicity to aquatic algae and cyanobacteria

Administrative Data

Short description of key information

The toxicity experiments revealed comparable toxicity for nanoparticulate ZnO, bulk ZnO, and ZnCl2, with a 72-h LC50 value near 60 µg/L, attributable solely to dissolved zinc

Key value for chemical safety assessment

EC50/LC50 for freshwater algae 60 µg/L

Discussion

A non-GLP/guideline conform study with non-OECD nanomaterial was conducted to characterize ZnO nanoparticles (nano-ZnOpowder and nano-ZnOdispersant) by using dynamic light scattering (DLS), transmission electron microscopy (TEM), and equilibrium dialysis. Both, ZnO nanoparticles and bulk ZnO showed rapid dissolution in freshwater medium (pH 7.6) in a similar manner. The chronic toxicity of ZnO nanoparticles was examined in Pseudokirchneriella subcapitata in comparison to the reference items, ZnCl2 and ZnO bulk. The toxicity experiments revealed comparable toxicity for nanoparticulate ZnO, bulk ZnO, and ZnCl2, with a 72-h LC50 value near 60 µg/L, attributable solely to dissolved zinc

Endpoint study record: Key.Nano.Franklin et al. (2007), Pseudokirchneriella

Administrative Data

other:Nano			
Purpose flag	key study		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	Non GLP/guideline conform study. Acceptable, well documented publication which meets basic scientific principles.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Franklin, N.M.	2007	Comparative Toxicity of Nanoparticulate ZnO, Bulk ZnO, and ZnCl2 to a Freshwater Microalga (Pseudokirchneriella subcapitata): The Importance of Particle Solubility	Environmental Science & Technology 41(24): 8484-8490					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Growth Inhibition Test with Pseudokirchneriella subcapitata using different concentrations of nano-ZnO solutions. Particle sizes of ZnO are ca. 30 nm. Effect concentrations are measured as elemental Zn. Besides toxicity also particle size distribution is evaluated. In comparison also bulk ZnO and ZnCl2 are tested.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	nanoparticulate ZnO
other: reference item	ZnO bulk
other: reference item	ZnCl2

Details on test material

- Name of test material: Nanoparticulate ZnO- nominal particle size: 30 nm
- Substance type: powder (= ZnOpowder) or aqueous form (= ZnOdispersant; containing 5% ZnO, 2%Teric N30 (= nonylphenol ethoxylate NPE), and 93% water)

Reference item:- ZnO bulk and ZnCl2

Analytical monitoring

yes

Details on analytical methods

IDENTIFICATION AND QUANTIFICATION OF TEST SUBSTANCE/PRODUCT
- All samples for zinc analysis were acidified (0.2%) with Tracepur HNO3 and measured by inductively coupled plasma-atomic emission spectrometry (Spectroflame, EOP, Kleve, Germany). The instrument was calibrated using a mixed metal standard (QCD Analysts, diluted to 2 mg/L) and matrix-matched standards, and spike recovery samples were included in all analyses. The detection limit for zinc was 0.5 μg/L.

Vehicle

no

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION
Concentrated suspensions of nanoparticulate ZnO (100 mg/L) were prepared and characterized using dynamic light scattering (DLS), transmission electron microscopy (TEM), and equilibrium dialysis. The concentration of ZnO chosen avoided a potential limitation in instrument detection by DLS and TEM and also ensured that excess zinc was present for the solubility experiments.
Dynamic Light Scattering (DLS): DLS measurements of nanoparticle suspensions were obtained using a highperformance particle sizer (Malvern Instruments Ltd., Worcestershire, U.K.). Appropriate amounts of nano-ZnOpowder or nano-ZnOdispersant were added to a synthetic freshwater algal medium, identical to that used for all algal bioassays. The low hardness and near-neutral pH of the medium was typical of natural freshwaters. All suspensions were vigorously shaken prior to analysis to break up visible clumps and resuspend any sedimented ZnO. The effect of employing a more thorough physical dispersion method on particle size distribution was assessed using an ultrasonicating probe (Daintree Scientific, St Helens, Australia; 2020XL; 60 W, 20 s duration). Samples were placed in clean disposable cuvettes, and at least three consecutive measurements were performed at 25°C, with each consisting of six runs of 20 s duration. Initially unfiltered suspensions were used to identify the entire particle size distribution. Samples were subsequently filtered through a 0.45 μm membrane filter to remove large particles that were interfering with the analysis. Samples that did not pass the instrument's internal quality criteria were disregarded.
Transmission Electron Microscopy (TEM): TEM (Philips CM100 operating at 80 kV) was used to visualize particle size and shape in subsamples of nanoparticle suspensions prepared in an identical manner to that outlined for DLS. Due to practical constraints, sonication and TEM sample preparation were undertaken 24 h prior to TEM analysis. Samples were prepared by depositing a drop (6 μL) of the suspensions on a carbon-coated copper specimen grid and allowing the water to evaporate in a laminar flow hood. All sample analyses included at least four different magnifications (10 000, 25 000, 46 000, and 96 000) and at least five fields of view. TEM samples from aqueous medium blanks (no nanoparticles added) were included as controls.Equilibrium Dialysis: Nanoparticle dissolution was assessed using Cole Parmer (Vernon Hills, IL) Spectra/Por 7 dialysis membranes of 1000 Da molecular weight cutoff (nominal pore size) and 45 mm diameter. The tubing was cut into 10 cm lengths and rinsed thoroughly in ultra-highpurity water (Milli-Q; Bedford, MA) prior to use. The dialysis cells were formed by filling the membrane tubes with Milli-Q water and sealing them with plastic dialysis clips which had been cleaned by soaking in 1% (v/v) nitric acid for several hours and thoroughly rinsed in Milli-Q water. The volume of the cells was approximately 10 mL. Test solutions were prepared by adding nano-ZnOpowder (100 mg/L) to a 0.01 M Ca(NO3)2 solution in Milli-Q water buffered to pH 7.5 with 2 mM piperazine-N,N′-bis(ethanesulfonic acid) (PIPES; Sigma-Aldrich, St Louis, MO). This medium was chosen to minimize adsorptive losses ofdissolved zinc onto container surfaces and to maintain the pH at 7.5 ± 0.15. Dialysis cells were added to the test solution and left to continually stir in a temperature- and light controlled incubator used for the algal bioassays.To minimize dilution effects, care was taken to

keep the volume of the dialysis cells below 5% of the total solution volume. At each sampling time, two cells were removed and sampled by pipet into polycarbonate vials. Samples of the external test solution were also taken at each time point for a measurement of pH and 0.1 µmfilterable zinc. Total zinc in the test was measured at the start and end of the experiment only. Dialysis experiments were also performed in an identical manner with two reference compounds (analytical grade), bulk ZnO (Ajax Finechem, Sydney, Australia), and Zn(NO3)2 (BDH, Poole, U.K.). To identify the influence of zinc impurities from the ZnO powders on the dialyzed zinc fraction, additional samples of nano-ZnOpowder and bulk ZnO were repeatedly washed with Milli-Q water and oven-dried, and new suspensions (100 mg/L) were prepared and measured for dissolved zinc (0.1 µm filterable) over the same sampling period.Algal Bioassay: Stock solutions (10, 50, and 100 mg/L of bulk ZnO and ZnCl2 were prepared in Milli-Q water. For ZnCl2, the stocks were acidified to pH < 2. For nano-ZnOpowder and nano-ZnOdispersant, stock solutions were prepared in the buffered algal test medium (pH 7.5) to match the characterization studies. Controls, together with with at least five contaminant concentrations (each in triplicate), were prepared with total zinc concentrations ranging from 25 to 600 µg Zn/L.

Test organisms

Test organisms (species)

Pseudokirchnerella subcapitata

Details on test organisms

TEST ORGANISM
- Source (laboratory, culture collection): American Type Culture Collection (Maryland, USA)
- Method of cultivation: cultured axenically in a U.S. Environmental Protection Agency (EPA) medium on a 24-h light cycle (Philips TL 40Wcool whitefluorescent lighting, Danvers, MA, 70 µmol photons/m2/s) at 24 °C.

Study design

Test type

static

Water media type

freshwater

Limit test

no

Total exposure duration

72 h

Test conditions

Hardness

15 mg/L

Test temperature

24°C

pH

7.5 ± 0.1

Nominal and measured concentrations

at least 5 concentrations between 25 - 600 µg Zn/L, and one control

Details on test conditions

TEST SYSTEM
- Test vessel:

- Material, size, headspace, fill volume: borosilicate glass minivials (30 mL)
- No. of vessels per concentration (replicates): 3
- No. of vessels per control (replicates): 3

GROWTH MEDIUM
- Standard medium used: yes
- Alkalinity: 9 mg/CaCO3

OTHER TEST CONDITIONS
- Photoperiod: 24-h light cycle
- Light intensity and quality: 70 µmol photons/m2/s

Any other information on materials and methods incl. tables

Algal Bioassay
Additional minivials were prepared at each concentration for pH measurement at the beginning and end of the test and for determination of the total and dissolved (0.1 µm filterable) zinc.
Exponentially growing cells of P. subcapitata were harvested by centrifuging (700g, 7 min) and were washed three times with the test medium. Each minivial was inoculated with a known concentration of prewashed cells to give aninitial cell density of (1–2) × 10^5 cells/mL. Algal cell counts were obtained using a four-color BDFACSCalibur (Becton Dickinson BioSciences, San Jose, CA) flow cytometer.
Chemical Analysis
All samples for zinc analysis were acidified (0.2%) with Tracepur HNO3 and measured by inductively coupled plasma-atomic emission spectrometry.The detection limit for zinc was 0.5 µg/L.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
72 h	IC50	68 µg/L	nominal	element total Zn (nano-ZnO powder)	growth rate (growth inhibition)	95% CL: 62-75 µg/L
72 h	IC50	49 µg/L	nominal	element total Zn (nano-ZnO dissolved)	growth rate (growth inhibition)	95% CL: 41-65 µg/L
72 h	IC50	68 µg/L	nominal	element dissolved Zn (0.1 µm filterable) (nano-ZnO dispersant)	growth rate (growth inhibition)	95% CL: 61-76 µg/L
72 h	IC50	44 µg/L	nominal	element dissolved Zn (0.1 µm filterable) (nano-ZnO dispersant)	growth rate (growth inhibiton)	95% CL: 36-62 µg/L

Any other information on results incl. tables

The current study using nanoparticulate ZnO (ca. 30 nm) has shown that small particles in aquatic systems and their bioavailability does not have to be significantly greater than that of larger particles. Particle characterization using transmission electron microscopy and dynamic light scattering techniques showed that particle aggregation is significant in a freshwater system, resulting in flocs ranging from several hundred nanometers to several microns. Chemical investigations using equilibrium dialysis demonstrated rapid dissolution of ZnO nanoparticles in a freshwater medium (pH 7.6), with a saturation solubility in the milligram per liter range, similar to that of bulk ZnO. Toxicity experiments using the freshwater alga Pseudokirchneriella subcapitata revealed comparable toxicity for nanoparticulate ZnO (nano-ZnOpowder and nano-ZnOdispersant), bulk ZnO, and ZnCl2, with a 72-h IC50 value near 60µg Zn/L, attributable solely to dissolved zinc. Care therefore needs to be taken in toxicity testing in ascribing

toxicity to nanoparticles per se when the effects may be related, at least in part, to simple solubility.

Applicant's summary and conclusion

Conclusions

The toxicity experiments revealed comparable toxicity for nanoparticulate ZnO, bulk ZnO, and ZnCl2, with a 72-h LC50 value near 60 µg/L, attributable solely to dissolved zinc

Executive summary

A non-GLP/guideline conform study with non-OECD nanomaterial was conducted to characterize ZnO nanoparticles (nano-ZnOpowder and nano-ZnOdispersant) by using dynamic light scattering (DLS), transmission electron microscopy (TEM), and equilibrium dialysis. Both, ZnO nanoparticles and bulk ZnO showed rapid dissolution in freshwater medium (pH 7.6) in a similar manner. The chronic toxicity of ZnO nanoparticles was examined in Pseudokirchneriella subcapitata in comparison to the reference items, ZnCl2 and ZnO bulk. The toxicity experiments revealed comparable toxicity for nanoparticulate ZnO, bulk ZnO, and ZnCl2, with a 72-h LC50 value near 60 µg/L, attributable solely to dissolved zinc

6.1.7 Toxicity to microorganisms

Endpoint summary: Toxicity to microorganisms

Administrative Data

Short description of key information

Based on nominal concentrations, the EC50 and EC20 value was greater than 1000 mg/L. The EC10 value was determined to be 750 mg/L for NM-110.

Key value for chemical safety assessment

EC50/LC50	1000 mg/L
EC10/LC10 or NOEC	750 mg/L

Discussion

BASF (2012)
The inhibitory effect of NM 110 (purity 99.1%) on activated sludge was investigated in a 180-min static test according to OECD 209 under GLP conditions. The activated sludge was taken from a municipal wastewater treatment plant. NM 110 was tested at concentrations of 62.5, 125, 250, 500 and 1000 mg/L. No details on test item preparation are specified. Based on nominal concentrations, the EC50 and EC20 value was greater than 1000 mg/L. The EC10 value was determined to be 750 mg/L.

Adams (2006)
Adams (2006) examined the antibacterial activity of the non-OECD NM (ZnO powder; mean particle size 480 nm) under non-GLP/guideline conform conditions. Bacillus subtilis or Escherichia coli were exposed to nominal concentrations of 0.04 – 21.3 mg/L for 6 hours in suspension and afterwards cultures were plated onto Luria-Bertani plates and left grow for 14-20 h. Test solutions were prepared in Milli-Q® water without sonification. E. coli was less sensitive to the addition of ZnO nanoparticles than B. subtilis.

Endpoint study record: WoE.Nano.BASFSE.08G0656/10G095.2012

Administrative Data

Purpose flag	weight of evidence
Study result type	experimental result
Reliability	1 (reliable without restriction)
Rationale for reliability incl. deficiencies	GLP and guideline conform study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	BASF SE	2012	NM-110 Zinc Oxide - determination of the Inhibition of Oxygen Consumtion in the Activated Sludge Respiration Inhibition Test		BASF SE, Experimental Toxicology and Ecology, Germany	08G0656/10G095	BASF SE	10/0656	2012-11-08

Data access

data submitter is data owner

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 209 (Activated Sludge, Respiration Inhibition Test)	

GLP compliance

yes (incl. certificate)

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
common name	NM-110

Details on test material

- Name of test material: NM-110 Zinc Oxide
- Physical state: Solid/ White
- Analytical purity: 99.1g/100 g
- Lot/batch No.: NM-110 Reference Nanomaterial

Test organisms

Test organisms (species)

activated sludge of a predominantly domestic sewage

Details on inoculum

- Laboratory culture:
Activated sludge from the municipal wastewater treatment plant of Mannheim, Germany was colledted from the aeration tank of the plant.
- Preparation of inoculum for exposure: The activated sludge suspension was sieved with a fine woven mesh (mesh size about 1 mm). This suspension was pre-aerated over night at room temperature. At the next day the sludge suspension was washed once with drinking water and the suspension was adjusted to 3 g/L dry matter.- Pretreatment: none- Initial biomass concentration: 1.5 g/L dry substance

Study design

Test type

static

Water media type

freshwater

Limit test

yes

Total exposure duration

180 min

Test conditions

Details on test conditions

TEST SYSTEM
- Test vessel: Glas-beakers (nominal volume 1L)
- Type (delete if not applicable): closed
- Aeration: the incubation was started by aeration of the test vessels with pressured air
- Type of flow-through (e.g. peristaltic or proportional diluter):
- No. of vessels per concentration (replicates): 2
- No. of vessels per control (replicates): 4
- No. of vessels per vehicle control (replicates): 2 for each reference substance concentration

OTHER TEST CONDITIONS
- Adjustment of pH: no

TEST CONCENTRATIONS
- Spacing factor for test concentrations: 2
- Justification for using less concentrations than requested by guideline:
- Test concentrations: 1000, 500, 250, 125, 62,5 mg/l

Reference substance (positive control)

yes (3,5 dichlorophenol)

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
180 min	EC10	720 mg/L	nominal	test mat.	respiration rate	391.8/1319.1
180 min	other: EC20	> 1000 mg/L	nominal	test mat.	respiration rate	
180 min	EC50	> 1000 mg/L	nominal	test mat.	respiration rate	

Results with reference substance (positive control)

The EC50 of the reference substance 3,5-dichlorophenol was in the range of 2-25 mg/L in 3 hours.

Any other information on results incl. tables

The value of effect concentration of EC10 was given with an accuracy of 2 significant digits. The degree of inhibition was evaluated by Probit analysis according to Finney.
The results in this study were consistent with the validity criteria with one exception. The mean oxygen uptake in the blank controls was lower than 20 mg/g*h (17 mg/g*h). Because the reference substance shows an EC50 in the specified range (usual range of EC50 in the
laboratory was 4.5 – 11.9 mg/L in the year 2012) and the measured oxygen uptake from the test substance concentrations showed a good curve progression the study is classified as valid. In the year 2012 the used activated sludge which was collected from the same waste water treatment plant showed a maximum oxygen consumption of 35.8 mg/g*h and minimum oxygen consumption of 10.5 mg/g*h.

Applicant's summary and conclusion

Conclusions

Based on nominal concentrations, the EC50 and EC20 value was greater than 1000 mg/L. The EC10 value was determined to be 750 mg/L for NM-110.

Executive summary

The inhibitory effect of NM 110 (purity 99.1%) on activated sludge was investigated in a 180-min static test according to OECD 209 under GLP conditions. The activated sludge was taken from a municipal wastewater treatment plant. NM 110 was tested at concentrations of 62.5, 125, 250, 500 and 1000 mg/L. No details on test item preparation are specified. Based on nominal concentrations, the EC50 and EC20 value was greater than 1000 mg/L. The EC10 value was determined to be 750 mg/L.

Endpoint study record: WoE.Nano.Adams et al. (2006), E. coli

Administrative Data

Purpose flag	weight of evidence		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	Non-GLP conform study. The study followed no guideline but is sufficient for assessment. Acceptable, well documented publication which meets basic scientific. Non-OECD material was used.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Adams, L.K.	2006	Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions	Water Research 40: 3527-3532					

Data access

data published

Cross-reference to same study

see 6.1.7., B. subtilis

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

The toxic effects associated with ZnO water suspensions using the gram negative E. coli in comparison to TiO2 and SiO2 are examined. Concentration at which the suspension is toxic to the test organisms are determined as well as the influence of size of the released nanoparticle on the antibacterial activity. Further it is determined whether natural light stimulates toxicity of the nanoparticles to bacteria.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	ZnO powder

Details on test material

- Name of test material: ZnO
- Substance type: powder, 420 - 640 nm (mean: 480 nm)
- Physical state: solid
- Analytical purity: no data

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: The ZnO powder was added to 100 ml of Milli-Q® water to obtain a final concentration of 10 g/L and shaken vigorously.

Test organisms

Test organisms (species)

Escherichia coli

Study design

Test type

other: test conducted with Petri plates

Limit test

no

Total exposure duration

6 h

Remarks bacteria were exposed to ZnO nanoparticles in suspension and afterwards cultures were plated onto LB plates and left to grow for 14-20 h

Post exposure observation period

Counting of bacteria colonies after growth on LB plates for 14-20 h

Test conditions

Test temperature

23°C average, if exposed to natural sunlight; 36°C during growth on LB plates

Nominal and measured concentrations

10, 50, 100, 500, 1000, 2000, 5000 ppm (0.04, 0.213, 0.42, 2.13, 4.26, 8.52, 21.3 mg/L) (nominal)

Details on test conditions

TEST SYSTEM
- replicates: 6

Any other information on materials and methods incl. tables

Organism cultivation: E. coli DH5α(courtesy of Dr. Charles Stewart, Rice University, Houston, TX) were maintained on Luria–Bertani (LB) plates. For all experiments, the bacteria were cultivated in a minimal Davis medium (MD). MD is a variation of Davis medium in which the potassiumphosphate concentration was reduced by 90%. This medium consisted of 0.7 g K_2HPO_4, 0.2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g Na-citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, and 1 g glucose in 1 L of Milli-Q® at pH 7.0. MD medium was chosen as the antibacterial test medium as previous research has shown that other nanosized aggregates precipitate out of suspension in media containing high phosphate concentrations.
Preparation of nanoparticle suspensions: ZnO (67 and 820nm advertised particle size) powder was obtained from Sigma-Aldrich (St. Louis, MO, USA). ZnO powder at 44 mm particle size was obtained from Alfa Aesar (Ward Hill, MA, USA). The advertised particle size was compared to the measured particle size in suspension. The powder was added to 100 mL of Milli-Q® water to obtain a final concentration of 10 g/L and shaken vigorously. The actual size of the particles in suspension in water and in MD was determined using a dynamic light scattering device (Brookhaven Instrument Corporation, Holtsville, NY, USA) for particles below 1 mm diameter, and optical microscopy (Nikon Optiphot, Japan) for those above this limit. All sizes were confirmed using TEM.
Assessment of toxicity to bacteria: Petri plates containing liquid MD media were supplemented with appropriate concentrations (10-5000 ppm) of nanoparticle suspensions to achieve a final volume of 5ml prior to inoculation with an overnight culture of E. coli (OD_{600} = 0.002). Antibacterial activity assays were conducted in the presence of sunlight with the small-sized particle suspensions. To obtain data on the effect of size and light on toxicity, suspensions were added at pre-determined toxic concentrations. Control plates were prepared containing only MD medium and bacteria. Plates were sealed with Parafilm (American National Can, Chicago, IL, USA) and wrapped in aluminium foil to simulate dark conditions

where required. All plates were placed on a rocker platform (Bell Company Biotechnology, Vineland, NJ, USA) to maintain the nanoparticles in suspension and left in direct sunlight for 6h (9AM to 3PM). The experiments were conducted in the window of a southeast facing laboratory on bright days (23°C average temperature, UV Index 6–7) in October in Houston, TX (291N, 951W). The average outdoor incident luminescence during the test periods was 50.4 klux/h, with the indoor values being similar, as the windows had no special coating. Cultures were diluted to achieve cell concentrations of approximately 1000 CFU/mL, spread onto LB plates, and left to grow at 36°C for 14 – 20 h. Colonies were counted and compared to control plates to calculate percentage growth inhibition. All treatments were prepared in duplicate and repeated on three separate occasions.

Results and discussions

Any other information on results incl. tables

Table: % growth inhibition when particle suspensions were applied in light at various concentrations (n.d. = not determined)

	Percentage growth inhibition at specified concentration (± SD, n=6)						
	10 ppm	**50 ppm**	**100 ppm**	**500 ppm**	**1000 ppm**	**2000 ppm**	**5000 ppm**
ZnO (480 nm)	14 ± 3.5	22 ± 6.5	28 ± 4.9	38 ± 8.9	48±7.7	n.d.	n.d.

Antibacterial activity increased with dose. The results showed that the Gram-negative E.coli was less sensitive to the addition of ZnO nanoparticles than Gram-positive B. subtilis, which was also tested (see cross-reference). Toxicity seems not to be related to particle size. E. coli was less susceptible to ZnO exposure than B. subtilis, with minimal growth inhibition under dark conditions, which might reflect differences in physiology, metabolism, or degree of contact.

Applicant's summary and conclusion

Executive summary

Adams (2006) examined the antibacterial activity of the non-OECD NM (ZnO powder; mean particle size 480 nm) under non-GLP/guideline conform conditions. Bacillus subtilis (see 6.1.7., B. subtilis) or Escherichia coli were exposed to nominal concentrations of 0.04 – 21.3 mg/L for 6 hours in suspension and afterwards cultures were plated onto Luria-Bertani plates and left grow for 14-20 h. Test solutions were prepared in Milli-Q water without sonification. E. coli was less sensitive to the addition of ZnO nanoparticles than B. subtilis.

Endpoint study record: WoE.Nano.Adams et al. (2006), B. subtillis

Administrative Data

Purpose flag	weight of evidence		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	Non-GLP conform study. The study followed no guideline but is sufficient for assessment. Acceptable, well documented publication/study report which meets basic scientific principles. Non-OECD material was used.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Adams, L.K.	2006	Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions	Water Research 40: 3527-3532					

Data access

data published

Cross-reference to same study

see 6.1.7., E. coli

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

The toxic effects associated with ZnO water suspensions using the grampositive B. subtilis in comparison to TiO2 and SiO2 are examined. Concentration at which the suspension is toxic to the test organisms are determined as well as the influence of size of the released nanoparticle on the antibacterial activity. Further it is determined whether natural light stimulates toxicity of the nanoparticles to bacteria.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	ZnO powder

Details on test material

- Name of test material (as cited in study report): ZnO
- Substance type: powder, 420 - 640 nm (mean: 480 nm)
- Physical state: solid
- Analytical purity: no data

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: The ZnO powder was added to 100 ml of Milli-Q® water to obtain a final concentration of 10

g/L and shaken vigorously.

Test organisms

Test organisms (species)

Bacillus subtilis

Study design

Test type

other: test conducted with Petri plates

Limit test

no

Total exposure duration

6 h

Remarks bacteria were exposed to ZnO nanoparticles in suspension and afterwards cultures were plated onto LB plates and left to grow for 14-20 h

Post exposure observation period

Counting of bacteria colonies after growth on LB plates for 14-20 h

Test conditions

Test temperature

23°C average, if exposed to natural sunlight; 36°C during growth on LB plates

Nominal and measured concentrations

10, 50, 100, 500, 1000, 2000, 5000 ppm (0.04, 0.213, 0.42, 2.13, 4.26, 8.52, 21.3 mg/L) (nominal)

Details on test conditions

TEST SYSTEM
- replicates: 6

Any other information on materials and methods incl. tables

Organism cultivation: B. subtilis CB310 (courtesy of Dr. Charles Stewart, Rice University, Houston, TX) were maintained on Luria–Bertani (LB) plates. For all experiments, the bacteria were cultivated in a minimal Davis medium (MD). MD is a variation of Davis medium in which the potassiumphosphate concentration was reduced by 90%. This medium consisted of 0.7 g K2HPO4, 0.2 g KH2PO4, 1 g (NH4)2SO4, 0.5 g Na-citrate, 0.1 g MgSO4 • 7H2O, and 1 g glucose in 1 L of Milli-Q® at pH 7.0. MD medium was chosen as the antibacterial test medium as previous research has shown that other nanosized aggregates precipitate out of suspension in media containing high phosphate concentrations.
Preparation of nanoparticle suspensions: ZnO (67 and 820nm advertised particle size) powder was obtained from Sigma-Aldrich (St. Louis, MO, USA). ZnO powder at 44 mm particle size was obtained from Alfa Aesar (Ward Hill, MA, USA). The advertised particle size was compared to the measured particle size in suspension. The powder was added to 100 mL of Milli-Q® water to obtain a final concentration of 10 g/L and shaken vigorously. The actual size of the particles in suspension in water and in MD was determined using a dynamic light scattering device (Brookhaven Instrument Corporation, Holtsville, NY, USA) for particles below 1 mm diameter, and optical microscopy (Nikon Optiphot, Japan) for those above this limit. All sizes were confirmed using TEM.
Assessment of toxicity to bacteria: Petri plates containing liquid MD media were supplemented with appropriate concentrations (10–5000 ppm) of nanoparticle suspensions to achieve a final volume of 5ml prior to inoculation with an overnight culture of B. subtilis (OD600 = 0.002). Antibacterial activity assays were conducted in the presence of sunlight with the small-sized particle suspensions. To obtain data on the effect of size and light on toxicity, suspensions were added at pre-determined toxic concentrations. Control plates were prepared containing only MD medium and bacteria. Plates were sealed with Parafilm

(American National Can, Chicago, IL, USA) and wrapped in aluminium foil to simulate dark conditions where required. All plates were placed on a rocker platform (Bell Company Biotechnology, Vineland, NJ, USA) to maintain the nanoparticles in suspension and left in direct sunlight for 6h (9AM to 3PM). The experiments were conducted in the window of a southeast facing laboratory on bright days (23°C average temperature, UV Index 6–7) in October in Houston, TX (291N, 951W). The average outdoor incident luminescence during the test periods was 50.4 klux/h, with the indoor values being similar, as the windows had no special coating. Cultures were diluted to achieve cell concentrations of approximately 1000 CFU/mL, spread onto LB plates, and left to grow at 36°C for 14 – 20 h. Colonies were counted and compared to control plates to calculate percentage growth inhibition. All treatments were prepared in duplicate and repeated on three separate occasions.

Results and discussions

Any other information on results incl. tables

Table: % growth inhibition when particle suspensions were applied in light at various concentrations (n.d. = not determined)

	Percentage growth inhibition at specified concentration (± SD, n=6)						
	10 ppm	50 ppm	100 ppm	500 ppm	1000 ppm	2000 ppm	5000 ppm
ZnO (480 nm)	90 ± 4.4	98 ± 0.8	98 ± 1.4	98 ± 0.8	n.d.	n.d.	n.d.

Antibacterial activity increased with dose. The results showed that the Gram-positive B. subtilis was more sensitive to the addition of ZnO nanoparticles than Gram-negative E. coli, which was also tested (see cross-reference). Toxicity seems not to be related to particle size. There was near-complete inhibition of B. subtilis growth (even at the lowest tested dose of 10 ppm) under both dark and illuminated conditions.

Applicant's summary and conclusion

Executive summary

Adams (2006) examined the antibacterial activity of the non-OECD NM (ZnO powder; mean particle size 480 nm) under non-GLP/guideline conform conditions. Bacillus subtilis or Escherichia coli (see 6.1.7, E.coli) were exposed to nominal concentrations of 0.04 – 21.3 mg/L for 6 hours in suspension and afterwards cultures were plated onto Luria-Bertani plates and left grow for 14-20 h. Test solutions were prepared in Milli-Q water without sonification. E. coli was less sensitive to the addition of ZnO nanoparticles than B. subtilis.

6.2 Sediment toxicity

Endpoint summary: Sediment toxicity

Administrative Data

Discussion

Fabrega and Galloway (2010)

An acute standard 10-day sediment toxicity test was conducted according to OSPARCOM1995/ASTM E1367-99 under GLP conditions. Thus, adult Corophium volutator (size range 4-7 mm, n = 20) were exposed to NM 112, NM 113 and ionic zinc (no details on purity). Dosing and exposures were done via water or via sediment. The method used to determine the endpoint is not specified. For all tested substances a concentration-dependent increase in mortality was observed in a similar manner. Acute exposure through the overlying water was 10-fold more toxic than acute exposure through the sediment.

Fabrega et al. (2011)
The organism Corophium volutator was administered over whole life cycle (100 d) with 0.2, 0.5 and 1 mg/L of NM-112 and the reference materials NM-113 and ionic Zn to investigate the effect on mortality, growth and reproductive endpoints. The study was performed according to OSPARCOM1995/ASTM E 1367-99 and under GLP conditions. The organism was treated in water (7 cm of sieved natural sediment and aerated seawater) and examined after 28, 63 and 100 days. For all test items, delayed growth and an affected reproductive outcome were observed. Solubility studies suggest that toxicity of NPs was not solely due to Zn2 + and the possible uptake of ZnO particles via other routes i.e dietary uptake might impact direct comparison between the exposures.. STEM-EDX analysis was used to characterize insoluble zinc precipitates (sphaerites) of high sulfur content, which accumulated in the hepatopancreas following exposures. The elemental composition of the sphaerites did not differ for ZnO NP, Zn2+, and bulk ZnO exposed organisms.

Endpoint study record: WoE.Fabrega (2010).Sediment toxicity (short-term)

Administrative Data

Purpose flag	weight of evidence		
Study result type	experimental result		
Reliability	2 (reliable with restrictions)		
Rationale for reliability incl. deficiencies	PARCOM1995/ASTM E1367-99 and GLP guideline study. Few details on the results. A graphic was attached showing a comparison of toxicity. However, the concentrations can not be clearly read from this graphic.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Julia Fabrega and Tamara Galloway	2010	Interim report for Ecotoxicology testing of manufactured ZnO and CeO2 nanoparticles	http://www.nanotechia-prospect.org/managed_assets/files/ecotox_interim_report.pdf	University of Exeter		University of Exeter		2010-10-01

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
equivalent or similar to	other guideline: PARCOM1995/ASTM E1367-99	

Principles of method if other than guideline

Acute sediment tests were based on standard 10-day sediment toxicity tests (ASTM, 2000; Roddie and Thain, 2001; USEPA, 2001) with a light regime modifications, as suggested by Scarlett et al. (2007). Adult C. volutator (size range 4–7 mm, n = 20) were exposed to increasing concentrations of ZnO MNP, zinc ions and bulk zinc. Dosing and exposures were done either via water or via sediment. The animals

were not fed during the test. At the end of the test the sediment was gently sieved (300 μm) and the number of alive, dead and missing amphipods in each vessel recorded.

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	Zinc oxide - Nanosun (= NM-112)
other: reference item	micro-sized ZnO (= NM-113)
other: reference item	ionic Zn

Details on test material

- Name of test material: Zinc oxide - nanosun (NM-112)
- Substance type: solid- Other: Partice size ~ 30 nm
Reference item:
- micro-sized ZnO (NM-113)- ionic Zn

Analytical monitoring

no

Vehicle

no

Test organisms

Test organisms (species)

Corophium volutator

Details on test organisms

TEST ORGANISM
- Common name:Corophium volutator
- Source:Otter estuary, Devon (ordinance survey grid reference: SY065820).
- Length at study initiation (length definition, mean, range and SD):4-7 mm
- Feeding during test No
- Food type:
- Amount:
- Frequency:
ACCLIMATION
- Acclimation period: 7-10 days
- Acclimation conditions (same as test or not): same as test conditions
- Type and amount of food: aquarium invertebrate food (Liquefy Marine, Interpret Ltd., Dorking, U.K.) and two drops
- Feeding frequency: weekly
- Health during acclimation (any mortality observed):
QUARANTINE (wild caught)
- Duration:
- Health/mortality:

Study design

Test duration type

short-term toxicity

Test type

static

Water media type

brackish water

Limit test

no

Total exposure duration

10 d

Post exposure observation period

No post exposure observation

Test conditions

Test temperature

12°C

pH

8

Salinity

25 ± 0.8 ppt

Details on test conditions

TEST SYSTEM
- Test vessel: 2L beakers
- Type (delete if not applicable): open
- Fill volume: 500 ml
- Aeration: Yes
- Type of flow-through (e.g. peristaltic or proportional diluter): N/A
- Renewal rate of test solution (frequency/flow rate):N/A
- No. of organisms per vessel: 20
- No. of vessels per concentration (replicates): 3
- No. of vessels per control (replicates):3
- No. of vessels per vehicle control (replicates): N/A
- Biomass loading rate:

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: Artifical seawater made in house
- Total organic carbon:
- Particulate matter:
- Metals:
- Pesticides:
- Chlorine:
- Alkalinity:
- Ca/mg ratio:
- Conductivity:
- Culture medium different from test medium:
- Intervals of water quality measurement:

OTHER TEST CONDITIONS

- Adjustment of pH:
- Photoperiod: 12 hours light to 12 h dark
- Light intensity:
EFFECT PARAMETERS MEASURED (with observation intervals if applicable) :
TEST CONCENTRATIONS
- Spacing factor for test concentrations:
- Justification for using less concentrations than requested by guideline:
- Range finding study
- Test concentrations:
- Results used to determine the conditions for the definitive study:

Any other information on materials and methods incl. tables

PREPARATION AND APPLICATION OF TEST SOLUTION
- Method: Experimental test concentrations were prepared following the protocol suggested by the National Physics Laboratory and available online at http://www.nanotechia-prospect.org/. In brief, a known concentration of MNP was measured out and made into a paste by addition of a drop or two of deionised water. Subsequently, 9 more drops of water were added to the paste and mixed prior to increasing the volume up to 15 ml with DDI water. The suspension was sonicated twice for 10 seconds (Cole-Parmer ® 130-Watt Ultrasonic Processors (50/60 Hz, VAC 220)) and used as the stock suspension. Known volumes of stock suspension were aliquoted out, added to the experimental beakers, at the required dosing regimes, and mixed with a glass rod.
- Controls: artifical seawater
Acute sediment tests were based on standard 10-day sediment toxicity tests (ASTM, 2000; Roddie and Thain, 2001; USEPA, 2001) with a light regime modifications, as suggested by Scarlett et al. (2007) . Adult C. volutator (size range 4–7 mm, n = 20) were exposed to increasing concentrations of ZnO MNP, zinc ions and bulk zinc. Dosing and exposures were done either via water or via sediment. The animals were not fed during the test. At the end of the test the sediment was gently sieved (300 μm) and the number of alive, dead and missing amphipods in each vessel recorded.
Water exposure verses sediment exposure.
Micro-size ZnO and ionic ZnO were used as reference material.

Results and discussions

Any other information on results incl. tables

Acute exposure (10 days) of C. Volutator to ZnO MNPs (modified ASTM test guideline 2000) caused a concentration-dependent increase in mortality which was similar for bulk (micron sized), soluble and nanoscale ZnO irrespective of particle size (average particle sizes tested: 30 -170 nm).
Acute exposure through the overlying water was 10-fold more toxic than acute exposure through the sediment. This would suggest an increase in bioavailability of ZnO MNPs when delivered through the water column, although this has not yet been confirmed.
See attached document for graph.

Overall remarks, attachments

Attached background material

Attached document	Remarks
A comparison of the toxicity (represented as survival (%) to adult C. volutator of 10 day waterborne.pdf / 104.32 KB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN1**	

Applicant's summary and conclusion

Conclusions

For all tested substances a concentration-dependent increase in mortality was observed in a similar

manner.

Executive summary

An acute standard 10-day sediment toxicity test was conducted according to OSPARCOM1995/ASTM E1367-99 under GLP conditions. Thus, adult Corophium volutator (size range 4-7 mm, n = 20) were exposed to NM 112, NM 113 and ionic zinc (no details on purity). Dosing and exposures were done via water or via sediment. The method used to determine the endpoint is not specified. For all tested substances a concentration-dependent increase in mortality was observed in a similar manner. Acute exposure through the overlying water was 10-fold more toxic than acute exposure through the sediment.

Endpoint study record: WoE.Fabrega (2011).Sediment toxicity (long-term)

Administrative Data

Purpose flag	weight of evidence
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	GLP conform and similar to guideline.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Julia Fabrega,*,† Ratna Tantra,‡ Aisha Amer,† Bjorn Stolpe,§ Jordan Tomkins,‡ Tony Fry,‡ Jamie R. Lead,§ Charles R. Tyler,† and Tamara S. Galloway*,†		Sequestration of Zinc from Zinc Oxide Nanoparticles and Life Cycle Effects in the Sediment Dweller Amphipod Corophium volutator	Environ. Sci. Technol	University of Exeter and Birmingham and Plymouth		University of Exeter		2011-12-08

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
equivalent or similar to	other guideline: PARCOM1995/ASTM E1367-99	yes (additional endpoints)

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	Zinc oxide - Nanosun (NM-112)
other: reference item	micro-sized ZnO (NM-113)
other: reference item	ionic Zn

Details on test material

- Name of test material (as cited in study report): Zinc oxide
- Nanosun (NM-112)
- Physical state: solid
- Other: particle size 30 nm

Reference items:
- micro-sized ZnO (NM-113) and ZnCl2

Analytical monitoring

yes

Vehicle

no

Test organisms

Test organisms (species)

Corophium volutator

Details on test organisms

TEST ORGANISM
- Common name: Corophium volutator
- Feeding during test: Yes
- Food type: aquarium invertebrate food (Liquefy Marine, Interpret Ltd., Dorking, U.K.).
- Amount: 2 drops
- Frequency: once a week

ACCLIMATION
- Acclimation period: 7-10 days
- Acclimation conditions (same as test or not): yes
- Type and amount of food: aquarium invertebrate food (Liquefy Marine, Interpret Ltd., Dorking, U.K.) and two drops
- Feeding frequency: once a week

Study design

Test duration type

long-term toxicity

Test type

semi-static

Water media type

brackish water

Limit test

yes

Total exposure duration

100 d

Remarks 23, 63 and 100 days

Post exposure observation period

No post exposure period

Test conditions

Hardness

N/A

Test temperature

12 +/- 0.6°C

pH

8+/- 0.2

Salinity

25+/-0.8 ppt

Details on test conditions

TEST SYSTEM
- Test vessel: 2L beakers
- Type (delete if not applicable):closed
- Material, size, headspace, fill volume: 500 ml fill volume
- Aeration: yes
- Type of flow-through (e.g. peristaltic or proportional diluter): N/A
- Renewal rate of test solution (frequency/flow rate): every 7 days and 24 h after feeding
- No. of organisms per vessel: 20 neonates
- No. of vessels per concentration (replicates): 3
- No. of vessels per control (replicates): 3
- No. of vessels per vehicle control (replicates): 0
- Biomass loading rate:

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water:
- Total organic carbon: 4.18 ± 1.15%.
- Salinity: 25 ppt

OTHER TEST CONDITIONS
- Adjustment of pH:
- Photoperiod: 12 h light:12 h dark
- Light intensity:

EFFECT PARAMETERS MEASURED (with observation intervals if applicable) :

VEHICLE CONTROL PERFORMED
- no (no vehicle used)

RANGE-FINDING STUDY
- Test concentrations: 0, 0.2, 0.5 and 1 mg/L
- Results used to determine the conditions for the definitive study:

Any other information on materials and methods incl. tables

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: Suspensions of ZnO NPs and bulk ZnO were prepared using standard operation protocols developed by the National Physics Laboratory (http://www.nanotechia-prospect.org). The NPs and bulk powders were made into a paste by adding one or two drops of deionized water (DIW) to 25 mg of particle mass and the mixture stirred with a metal spatula. Using a glass Pasteur pipet, 9−10 drops of DIW were then added slowly, continuing to mix the paste. The remaining DIW (250 mL, except for the few drops used to make the paste) was added to the paste and the suspension sonicated twice for 10 s using an

ultrasonic probe (Cole Parmer 130 W Ultrasonic Processor). Bulk and ZnO NM suspensions were made freshly, immediately before dosing the exposure vessels.
- Controls: Artifical seawater
Micro-scale ZnO reference substance and ZnCl2 reference substance are used to compare against nano-scale ZnO.
The sizes of individual discrete particles (circular diameter) determined by TEM. The crystallite diameter determined by the Scherrer's equation from X-ray diffraction (XRD). Dynamic light scattering (DLS) measurements were made.
Transmission Electron Microscopy and Energy Dispersive X-ray Analysis. The chemical composition of the granules formed in the hepatopancreas of the organisms after exposure for 100 days to the different forms of zinc (ZnO NP, bulk ZnO, and Zn ions) was investigated by scanning transmission electron microscopy (STEM) and energydispersive X-ray spectroscopy (EDX, Jeol 7000F with Oxford Inca EDX).

Chronic Exposure to ZnO Nanoparticles

C. volutator neonates were collected from the acclimated laboratory stock cultures by sieving the sediment though a 500 μm nominal pore size sieve to remove larger animals. The sieved fraction was then resieved at 300 μm to collect the neonates (0.6−1.2 mm in length). For each test vessel, 20 neonate organisms were transferred to 2 L glass beakers containing about 7 cm of sieved natural sediment and 500 mL of continuously aerated seawater (pH 8 ± 0.2, 25 ± 0.8 ppt, 12 ± 0.6 °C). Dosing of ZnO NP, bulk ZnO, and the same mass of Zn ions (from ZnCl2) was conducted via the water. Organisms were fed weekly with two drops of aquarium invertebrate food (Liquefy Marine, Interpret Ltd., Dorking, U.K.). Water changes and redosing via the water occurred every 7 days and 24 h after feeding. A total of nine vessels were set up for each treatment regime (including for controls), and three replicate vessels were sampled from each treatment at 23, 63, and 100 days.

Results and discussions

Details on results

- Mortality of parent animals: <40% under all conditions
- Body lengthparent animals: 5.11 */- 0.84 mm
- Other biological observations: Specific growth rate and age of sexual differentiation were significantly delayed by increasingconcentrations of zinc, but after 100 days all populations were sexually differentiated. Sublethal toxicity (DNA damage in hemolymph cells) was significantly lower for nanosun ZnO (≥0.5 mg l-1) than for all other forms of zinc. This suggests that ZnO MNPs may have lower bioavailability than micron or soluble Zn to sediment dwelling organisms.

Reported statistics and error estimates

Anova and Bonferroni test

Any other information on results incl. tables

Toxicity of ZnO NPs

Survival (%) of C. volutator after 28, 63 and 100 days to a waterborne exposure to different forms of Zn (nanoparticle (MNP), bulk and soluble (Zn2+) and concentrations (0.2, 0.5 and 1 mg l-1). All forms of zinc effected the survival of C. volutator, and the effects were the greatest at the highest exposure concentration and after 100 days from the onset of exposure (ANOVA, $p < 0.05$).

	Treatment (mg l^{-1})									
	Control	**MNP 0.2**	**MNP 0.5**	**MNP 1**	**Bulk 0.2**	**Bulk 0.5**	**Bulk 1**	**Zn^{+}0.2**	**Zn^{+}0.5**	**Zn^{+}1**
DAYS										
28	88.75±2.5	80±14.1	83.3±12.6	93.3±5.8	82.5±3.5	92.5±3.5	92.5±3.5	96.7±5.8	88.4±5.8	85±8.7
63	85±4.1	87.5±10.6	86.7±11.5	86.7±15.3	81.6±27.5	87.5±3.5	80±22.9	80±21.8	80±5.0	86.7±7.6
100	91.7±5.8	72.5±3.5	78.3±5.8	66.7±10.4	71.6±10.4	78.3±7.6	63.3±16.1	56.7±10.4	70±5.0	63.3±10.4

Specific growth rates of C. volutator exposed to different concentrations and forms of zinc over 100 days. Specific growth rate was significantly reduced 23 days after the onset of exposure, with the highest concentrations of ZnO nanosun, micro-sized ZnO, and Zn+ causing about 11, 12, and 21% reduction of growth, respectively, from unexposed populations. After 63 days from the onset of exposure, only the populations exposed to micro-sized ZnO and Zn+ showed a slower growth rate. After 100 days, in most populations individuals had reached adult size The one exception to this was the population exposed to 1 mg L−1 bulk ZnO, where the organisms were still significantly smaller (ANOVA, $p < 0.05$).

Treatment	**28 days**	**63 days**	**100 days**
MNP0.2	13.34	0.44	2.46
MNP0.5	16.09	5.03	2.97
MNP1	10.82	7.94	5.45
Bulk0.2	-0.30	11.68	0.37
Bulk0.5	8.11	1.48	3.00
Bulk1	13.00	12.56	9.25
ZnCl20.2	3.92	10.64	3.19
ZnCl20.5	15.30	13.27	3.92
ZnCl21	20.36	17.51	4.39

Solubility studies suggested that toxicity of NPs was not solely due to Zn2+.

STEM-EDX analysis

The elemental composition of the sphaerites did not differ for ZnO NP, Zn2+, and bulk ZnO exposed organisms

Particle characteristics in artifical seawater was also determined

The sizes of individual discrete particles (circular diameter) determined by TEM were 35 ± 10 nm (n = 316) for ZnO NPs and 160 ± 81 nm (n = 204) for bulk ZnO, while the crystallite diameter determined by the Scherrer's equation from X-ray diffraction (XRD) data was 24.1 nm for the ZnO NP and 41.5 nm for the bulk ZnO. In both media, both types of particles were largely present as aggregates, as indicated by the TEM and by dynamic light scattering (DLS) measurements, showing high polydispersity indexes and hydrodynamic diameters that were considerably larger than the sizes of the discrete particles. Moreover, the hydrodynamic diameters were larger in seawater (670 ± 31 nm for NPs, 770 ± 32 nm for bulk) than in DIW (196 ± 8.4 nm for NPs, 390 ± 23 nm for bulk). The zeta potential DIW was positive for both ZnO NPs (+17 ± 2.1 mV) and bulk ZnO (+13.9 ± 0.66 mV) but negative in seawater (−10 ± 2.6 mV and −13 ± 1.9 mV, respectively). Thus, the isoelectric point is found at a pH between that DIW (typically pH 6) and seawater (pH 8), and therefore, pH is likely to play an important role in the stability of ZnO particles.

Overall remarks, attachments

Attached background material

Attached document	Remarks
Fabrega et al. 2011.pdf / 1.53 MB (application/octet-stream): **ENV/JM/MONO(2015)15/ANN17**	

Applicant's summary and conclusion

Conclusions

For all test items, delayed growth and an affected reproductive outcome were observed. The elemental composition of the sphaerites did not differ for ZnO NP, Zn2+, and bulk ZnO exposed organisms.

Executive summary

The organism Corophium volutator was administered over whole life cycle (100 d) with 0.2, 0.5 and 1 mg/L of NM-112 and the reference materials NM-113 and ionic Zn to investigate the effect on mortality, growth and reproductive endpoints. The study was performed according to OSPARCOM1995/ASTM E 1367-99 and under GLP conditions. The organism was treated in brackish water(7 cm of sieved natural sediment and aerated seawater)and examined after 28, 63 and 100 days. For all test items, delayed growth and an affected reproductive outcome were observed. Solubility studies suggest that toxicity of NPs was not solely due to Zn2+ and the possible uptake of ZnO particles via other routes i.e dietary uptake might impact direct comparison between the exposures.STEM-EDX analysis was used to characterize insoluble zinc precipitates (sphaerites) of high sulfur content, which accumulated in the hepatopancreas following exposures. The elemental composition of the sphaerites did not differ for ZnO NP, Zn2+, and bulk ZnO exposed organisms.

6.3 Terrestrial toxicity

Endpoint summary: Terrestrial toxicity

Administrative Data

Discussion

Toxicity to terrestrial plants

The toxicity and uptake of ZnO nanoparticles (particle size 20 nm, purity 99.5%) was studied in Lolium

perenne under non-GLP/guideline conform conditions (Lin and Xing, 2008). ZnO nanoparticles were characterized by TEM and BET analysis. Phytotoxicity experiment included three treatments; no treatment, treatment with ZnO nanoparticles or ZnSO4 heptahydrate solution, respectively. Seedling growth in both treatments was retarded with shorter roots and shoots compared to the control. In addition the seedling biomass decreased with increasing concentrations. The nominal IC50 (12 d) of ZnO nanoparticles was 64 mg/L (corresponding to 51 mg/L Zn), which was in a similar range compared to the IC50 value of ionic Zn. Furthermore, shrank morphology of the roots tips (epidermis and rootcap were broken, cortical cells were highly vacuolated and collapsed). It was also observed that ZnO nanoparticles were able to concentrate in the rhizosphere and enter the root cells. The authors suggested the the phytotoxicity of ZnO nanoparticles could not primarily come from their dissolution.

Lin and Xing (2007) also investigated the effect of ZnO nanoparticles (size: 20 nm, purity > 99.5%) on seed germination and root growth under non-GLP/guideline conform conditions. Seeds were soaked in deionized water, nanoparticle suspension or ionic Zn solution for about 2 h. The results indicated that seed germination of ryegrass and corn was inhibited by ZnO nanoparticles. Additionally, ZnO nanoparticles inhibited root growth of corn and practically terminated root development of the plants used. To test the phytotoxicity of ionic Zn in the suspension of ZnO nanoparticles, their supernatant was used and the Zn2+ concentration was determined by using ICP-OES. Equivalent concentrations made from ZnSO4 heptahydrate served as reference. Since no phytotoxicity was observed, the authors assumed that the phytotoxicity of ZnO nanoparticles was not directly from their dissolution in bulk aqueous solutions.

In conclusion, ZnO nanoparticles seemed to lead to retarded growth of terrestrial plants and decreased seed germination. However, the validity of the observed effects are considered questionable as apparently Zn ion effects were observed and the occurrence of agglomerates were not addressed.

6.3.1 Toxicity to soil macroorganisms except arthropods

Endpoint study record: Disregarded.Nano.Unrine et al. (2008), Eisenia

Administrative Data

Purpose flag	disregarded study		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	Extended abstract. Not sufficient for assessment due to missing information.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Unrine, J. et al.	2008	Spatial distribution and speciation of Au and Zn in terrestrial organisms exposed to Au and ZnO nanoparticles. Presented before the Division of Environmental Chemistry American Chemical Society, New Orleans, LA April 6-10, 2008	American Chemical Society, Preprints of Extended Abstracts 48(1): 334-340					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Exposure of earthworms to nanoparticle ZnO containing artificial soil followed by trace element analysis of earthworm tissue in order to evaluate spatial distribution of uptaken Zn. In comparison also ZnCl2 is tested.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5

Details on test material

- Name of test material (as cited in study report): colloidal ZnO in aqueous suspension
- Physical state: solid, nanoparticles of 1.2 nm (hydrodynamic diameter)
- Analytical purity: no data

Test organisms

Test organisms (species)

Eisenia fetida

Animal group

annelids

Study design

Study type

laboratory study

Test duration type

long-term toxicity

Substrate type

artificial soil

Limit test

no

Total exposure duration

28 d

Test conditions

pH

6 ± 0.5

Moisture

30% deionised water

Nominal and measured concentrations

1000 mg Zn/kg soil as ZnO (nanoscale) (nominal)

Details on test conditions

TEST SYSTEM
- Test container (material, size):
- Amount of soil or substrate:
- No. of organisms per container (treatment):
- No. of replicates per treatment group:
- No. of replicates per control:
- No. of replicates per vehicle control:

SOURCE AND PROPERTIES OF SUBSTRATE (if soil)
- Composition (if artificial substrate): 60% silica sand, 10% peat, 30% Kaolin- pH adjustment with crushed limestone

OTHER TEST CONDITIONS

- Photoperiod:
- Light intensity:
EFFECT PARAMETERS MEASURED (with observation intervals if applicable) :
VEHICLE CONTROL PERFORMED: yes/no
TEST CONCENTRATIONS
- Spacing factor for test concentrations:
- Justification for using less concentrations than requested by guideline:
- Range finding study
- Test concentrations:
- Results used to determine the conditions for the definitive study:

Any other information on materials and methods incl. tables

Controls used: deionised water or sodium acetate.
Earthworms were allowed to void their gut contents on moistened filter paper for 24h post exposure.
Segments of the earthworm bodies were fixed in formalin, embedded in hydrophilic melamine resin and sectioned to approximately 100 μm for subsequent analysis by μSXRF.
Trace Element Analysis: The uptake and spatial distribution of Zn in E. fetida was examined using spatially resolved μSXRF at beamline X26A, National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY USA. The Zn Kα fluorescence was recorded for 3s at each 10 x 10 μm pixel using a nine element Ge array detector while the sample was translated in a raster pattern. The intensities of Ti Kα and Ca Kα were also collected to show the outline of the earthworm and to indicate the location of any soil particles remaining in the gut. The spatial distribution of the fluorescence intensities was then plotted in two dimensions and full x-ray fluorescence spectra were recorded with an integration time of 300s at select pixels. In addition, for pixels of high intensity, we determined x-ray absorption near edge structure (XANES) and collected XRD diffraction patterns using an area XRD detector (Burker Axis). Because of the time and cost requirements of performing these advanced techniques, only a few (2-5) individuals from each treatment have been analyzed; however, efforts to analyze tissues from a greater number of individuals are ongoing.
Molecular Distribution of Zn in E. fetida: Proteins were extracted from E. fetida by homogenizing whole bodies in ice-cold 100 mM Tris, pH 7.2 using a pre-chilled glass micro-tissue grinder. Macromolecules in the crude extracts were separated by size using a size exclusion column (Superdex 200 HR, Amersham Biosciences) using 100 mM Tris, pH 7.2 as the mobile phase pumped at a rate of 0.5 mL/min. The ICP-MS was used as an element specific detector to detect Zn containing macromolecules by measuring the signal intensity at m/z 66 versus time. The column was calibrated using a molecular marker kit that contained a number of metal or heteroatom containing proteins. The proteins were detected using both ultraviolet absorbance at 210 nm or ICP-MS in order to create a calibration curve for estimating molecular mass based on retention time. The total and excluded volumes of the column were determined using blue dextran (2,000 kDa) and acetone (58.08 Da).

Results and discussions

Any other information on results incl. tables

Patterns of Zn distribution in E. fetida tissues were very similar among Zn treated animals, but the patterns of distribution differed markedly between Zn treated animals and control animals. While Zn treated animals had relatively homogenous distribution of Zn, control animals had loci of extremely high Zn concentrations and they were likely composed of a very high percentage of Zn. These loci produced very intense diffraction patterns indicating that they were composed of crystalline materials. We were not successful at identifying the crystalline substance(s) by comparison to diffraction patterns of known Zn containing substances. Total Zn concentrations, as determined by acid dissolution and analysis by ICP-MS were similar in earthworms regardless of treatment. We also observed that the control earthworms scavenged nearly all of the Zn from the control soil, which contained approximately 20 mg/kg prior to the

exposure and non-detectable quantities of Zn after exposure. The distribution of Zn among macromolecules was also similar among Zn-treated earthworms, but some control earthworms had very high concentrations of Zn bound to a ~10 kDa protein, suggesting that large amounts of Zn-metallothionein were present. The fact that the spatial and molecular distribution of Zn differed between Zn-treated animals and control animals but not among Zn treatments, suggests that Zn from ZnO may have been bioavailable.

Endpoint study record: Disregarded.Nano.Unrine et al. (2008), Caenorhabditis

Administrative Data

Purpose flag	disregarded study		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	Extended abstract. Not sufficient for assessment due to missing information.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Unrine, J. et al.	2008	Spatial distribution and speciation of Au and Zn in terrestrial organisms exposed to Au and ZnO nanoparticles. Presented before the Division of Environmental Chemistry American Chemical Society, New Orleans, LA April 6-10, 2008	American Chemical Society, Preprints of Extended Abstracts 48(1): 334-340					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Exposure of nematodes to nanoparticle ZnO containing medium followed by trace element analysis of nematode tissue in order to evaluate spatial distribution of uptaken Zn. In comparison also ZnCl2 is tested.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5

Details on test material

- Name of test material (as cited in study report): colloidal ZnO in aqueous suspension
- Physical state: solid, nanoparticles of 1.2 nm (hydrodynamic diameter)
- Analytical purity: no data

Details on preparation and application of test substrate

Nematodes (C. elegans) were exposed to ZnO in acetate buffered K medium (32 mM KCl, 51 mM NaCl, 150 mM acetate, pH 6)

Test organisms

Test organisms (species)

Caenorhabditis elegans

Animal group

nematods

Study design

Study type

laboratory study

Test duration type

short-term toxicity

Substrate type

other: ZnO in acetate buffered K medium

Limit test

no

Total exposure duration

24 h

Test conditions

pH

6

Nominal and measured concentrations

100 or 500 mg Zn/L as ZnO

Any other information on materials and methods incl. tables

After exposure nematodes were fixed in formalin for analysis by µSXRF.
The exposure concentrations were selected based on the observed LC50 value for ZnO (789 mg Zn/L).
Trace Element Analysis: The uptake and spatial distribution of Zn in C. elegans was examined using spatially resolved µSXRF at beamline X26A, National Synchrotron Light Source, Brookhaven National

Laboratory, Upton, NY USA. The Zn Kα fluorescence was recorded for 3s at each 10 x 10 µm pixel using a nine element Ge array detector while the sample was translated in a raster pattern. The spatial distribution of the fluorescence intensities was then plotted in two dimensions and full x-ray fluorescence spectra were recorded with an integration time of 300s at select pixels. In addition, for pixels of high intensity, we determined x-ray absorption near edge structure (XANES) and collected XRD diffraction patterns using an area XRD detector (Burker Axis).

Results and discussions

Any other information on results incl. tables

The patterns of Zn distribution depended on Zn exposure concentration. Control animals had regions of maximum Zn intensity in the anterior region of the body and these regions of maximal intensity spread towards the anterior end of the body with increasing exposure concentration from 100 to 500 mg Zn/L. The maximum Zn Kα intensities in the ZnCl2 exposed nematodes (not reported here) were nearly twice as great as in the ZnO exposed nematodes suggesting decreased bioavailability of Zn as ZnO relative to aqueous Zn ions. The fact that the LC50 values of these two forms of Zn were similar despite decreased bioavailability of Zn in nano-ZnO form, suggests that the two forms of Zn elicit toxicity by differing mechanisms. The regions of high Zn intensity did not produce diffraction patterns for any treatment; however, if crystalline materials were present, a detectable diffraction pattern would not be expected at such low concentrations.

6.3.2 Toxicity to terrestrial arthropods

Endpoint study record: Disregarded.Nano.Wan and Gong (2005), Epitrimerus

Administrative Data

Purpose flag	disregarded study		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	Documentation insufficient for assessment. Original reference in foreign language (Chinese)		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Wan, S. Q. and Gong, Z.N.	2005	Effect of Action of Mixture of Two Nano Particles with Two Insecticides to Pest Mite (Epitrimerus pyri)	Chinsese Journal of Pesticides 44(12): 570-572					

Data access

data published

Materials and methods

Application method

other: dipping

Principles of method if other than guideline

In comparison to two actives of insecticides (cypermethrin and alpha-terthienyl) on their own and these actives mixed with ordinary sized zinc and copper oxides, the synergistic actions for mixtures, cypermethrin and alpha-terthienyl with nano-sized zinc oxide and copper oxide, on pear rust mites were evaluated.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5

Details on test material

- Name of test material (as cited in study report): nano zinc oxide
- Analytical purity: 99.9%
- Median size: 30 nm
-Range of particle size: ≤ 80 nm)

Further test substances:

- Nano copper oxide (median particle size: 25 nm, range of particle size: ≤ 60 nm, analytical purity: 99.9%)
- Zinc oxide (range of particle size: 45 - 75 µm, analytical purity: ≥ 99.0%)
- Copper oxide (range of particle size: 150 µm, analytical purity: ≥ 99.0%)
- Cypermethrin (99%, Jinagmeng Pesticide, China)
- Alphaterthienyl (sigma)

Analytical monitoring

no

Vehicle

yes

Details on preparation and application of test substrate

- Test medium preparation: The actives were dissolved in a small amount of acetone (≤ 1%) and emulsified with 5 - 10% of polysorbate-80. Following this, serial dilutions of the test media were prepared with deionised water.
- Controls: 1% acetone
- Chemical name of vehicle (organic solvent, emulsifier or dispersant): 1% acetone as vehicle and 5 - 10% polysorbate 80 as emulsfier
- Concentration of vehicle in test medium (stock solution and final test solution): No data
- Evaporation of vehicle before use: No data

Test organisms

Test organisms (species)

other: Epitrimerus pyri

Animal group

Acari (leaf-dwelling predatory mite)

Details on test organisms

TEST ORGANISM
- Common name: Pear rust mite
- Source: Leaves of green jujube (South China Agricultural University)
- Date of collection: no data
- Cultural background: No application of any pesticides for 6 months.
- Disease free: no data

Study design

Study type

laboratory study

Test duration type

short-term toxicity

Total exposure duration

48 h

Remarks After the treatments with the test media for 5 seconds, the leaves with mites were incubated for 24 and 48 hours.

Post exposure observation period

24 or 48 hours

Test conditions

Test temperature

25 ± 1 °C

Humidity

80%

Nominal and measured concentrations

not given in the publication.

Details on test conditions

TEST SYSTEM
- Test container / cage (material, size): Petri dishes (9 cm)
- No. of replicates per treatment group: 3
- No. of replicates per per control / vehicle control: 3

EFFECT PARAMETERS MEASURED (with observation intervals if applicable) : Mortality
VEHICLE CONTROL PERFORMED: yes
TEST CONCENTRATIONS
- Spacing factor for test concentrations: No data- Range finding study
- Test concentrations: The data were not given in the publication

Reference substance (positive control)

no

Any other information on materials and methods incl. tables

The leaves of green jujube with mites were collected and immersed in the test media for 5 seconds. The leaves were placed in petri dishes and incubated at 25 °C for 24 and 48 hours, respectively. Especially, after 12 hours incubation, the leaves treated with alpha-terthienyl and its mixed agents of two nano-particles were irradiated under UV (wavelength: 340 - 400 nm, intensity: 15 w/cm2) for 3 hours. Then the leaves were incubated at 25 °C for further 24 and 48 hours, respectively. The mortality of mites attached to the leaves was determined at the end of incubations.

Results and discussions

Effect concentrations

Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
24 h	LC50	445.6 mg/L			mortality	
48 h	LC50	374.34 mg/L			mortality	
24 h	LC50	38.83 mg/L			mortality	mixture of nano-zinc oxide and cypermethrin (1:4)
48 h	LC50	14.18 mg/L			mortality	mixture of nano-zinc oxide and cypermethrin (1:4)
24 h	LC50	11.44 mg/L			mortality	mixture of nano-zinc oxide and alpha-terthienyl (1:4)
48 h	LC50	9.22 mg/L			mortality	mixture of nano-zinc oxide and alpha-terthienyl (1:4)

Any other information on results incl. tables

According to the authors the actives Cypermethrin and alpha Terhienyl mixed with nano-particled zinc oxide and copper oxide showed syntergistic effects to the tested mite.

Test substance	24 hours		48 hours	
	LC50 (mg/L)	CTC	LC50 (mg/L)	CTC
Nano-Zinc oxide	445.60		374.34	
Nano-copper oxide	866.55		478.65	
Cypermethrin	63.80		42.20	
Alpha-terthienyl	13.02		5.16	
Nano-Zinc oxide+ cypermethrin (1:4)	38.83	198.28	14.18	403.96
Nano-Copper oxide + cypermethrin (1:4)	55.93	140.01	20.46	281.42
Nano-Zinc oxide + alpha-terthienyl (1:4)	11.44	145.54	9.22	69.75

6.3.3 Toxicity to terrestrial plants

Endpoint summary: Toxicity to terrestrial plants

Administrative Data

Discussion

The toxicity and uptake of ZnO nanoparticles (particle size 20 nm, purity 99.5%) was studied in Lolium perenne under non-GLP/guideline conform conditions (Lin and Xing, 2008). ZnO nanoparticles were characterized by TEM and BET analysis. Phytotoxicity experiment included three treatments; no treatment, treatment with ZnO nanoparticles or ZnSO4 heptahydrate solution, respectively. Seedling

growth in both treatments was retarded with shorter roots and shoots compared to the control. In addition the seedling biomass decreased with increasing concentrations. The nominal IC50 (12 d) of ZnO nanoparticles was 64 mg/L (corresponding to 51 mg/L Zn), which was in a similar range compared to the IC50 value of ionic Zn. Furthermore, shrank morphology of the roots tips (epidermis and rootcap were broken, cortical cells were highly vacuolated and collapsed). It was also observed that ZnO nanoparticles were able to concentrate in the rhizosphere and enter the root cells. The authors suggested that the phytotoxicity of ZnO nanoparticles could not primarily come from their dissolution.

Lin and Xing (2007) also investigated the effect of ZnO nanoparticles (size: 20 nm, purity > 99.5%) on seed germination and root growth under non-GLP/guideline conform conditions. Seeds were soaked in deionized water, nanoparticle suspension or ionic Zn solution for about 2 h. The results indicated that seed germination of ryegrass and corn was inhibited by ZnO nanoparticles. Additionally, ZnO nanoparticles inhibited root growth of corn and practically terminated root development of the plants used. To test the phytotoxicity of ionic Zn in the suspension of ZnO nanoparticles, their supernatant was used and the Zn2+ concentration was determined by using ICP-OES. Equivalent concentrations made from ZnSO4 heptahydrate served as reference. Since no phytotoxicity was observed, the authors assumed that the phytotoxicity of ZnO nanoparticles was not directly from their dissolution in bulk aqueous solutions.
In conclusion, ZnO nanoparticles seemed to lead to retarded growth of terrestrial plants and decreased seed germination. However, the validity of the observed effects are considered questionable as apparently Zn ion effects were observed and the occurrence of agglomerates were not addressed.

Endpoint study record: WoE.Nano.Lin and Xing (2008), ryegrass root uptake

Administrative Data

Purpose flag	weight of evidence; robust study summary		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	This study did not follow an OECD guideline and was not performed under GLP conditions. Validity of the observed effects are questionable. Non-OECD material.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Lin, D. and Xing, B.	2008	Root uptake and phytotoxicity of ZnO nanoparticles	Environmental Science and Technology 42: 5580-5585					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Cell internalization and upward translocation of ZnO nanoparticles by Lolium perenne were examined.

The dissolution of ZnO nanoparticles and its contribution to the toxicity on ryegrass were also investigated. Zn2+ ions were used to compare and verify the root uptake and phytotoxicity of ZnO nanoparticles in a hydroponic culture system. The root uptake and phytotoxicity were visualised by light, scanning electron, and transmission electron microscopies.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	ZnO nanoparticles
other: reference material	ZnSO4 heptahydrate

Details on test material

- Name of test material: ZnO nanoparticles
- Physical state: solid, 20 ± 5 nm
- Analytical purity: 99.5%

Reference item:- ZnSO4 heptahydrate

Details on test substrate

Compositions of the nutrient solution (1 strength Hoagland solution): 20 ppm (NH4)2SO4, 10 ppm NH4NO3, 3.1 ppm NaH2PO4, 40 ppm K2SO4, 15 ppm CaCl2.2H2O, 0.35 ppm EDTA.FeNa.3H2O, 25 ppm MgSO4.3H2O, 20 ppm Al2(SO4)3.18H2O, 0.1 ppm ZnSO4.7H2O, 0.1 ppm H3BO3, 0.025 ppm CuSO4.5H2O, 1 ppm MnSO4.H2O and 0.05 ppm Na2MoO4.2H2O. All of these compositions were analytical grade with a purity of > 98%. The pH value of the nutrient solution was adjusted to near neutral.

Test organisms

Test organisms

Species	Lolium perenne
Plant group	Monocotyledonae (monocots)
Details on test organisms	- Common name: ryegrass - Plant family: Poaceae

Study design

Study type

laboratory study

Test duration type

short-term toxicity

Test type

other: root uptakeof ZnO

Substrate type

other: nutrient solution

Limit test

no

Total exposure duration

12 d

Test conditions

Test temperature

25-30°C in daytime (16 h), 15-20°C in nighttime (8 h)

pH

7

Nominal and measured concentrations

10, 20, 50, 100, 200, 1000 mg/L (nominal), control

Details on test conditions

TEST SYSTEM
- Test container (type, material, size): 1000 mL beakers
- No. of seeds per container: 9
- No. of replicates per treatment group: 3

NUTRIENT MEDIUM (if used)
- Description: 20 ppm (NH4)2SO4, 10 ppm NH4NO3, 3.1 ppm NaH2PO4, 40 ppm K2SO4, 15 ppm CaCl2.2H2O, 0.35 ppm EDTA.FeNa.3H2O, 25 ppm MgSO4.3H2O, 20 ppm Al2(SO4)3.18H2O, 0.1 ppm ZnSO4.7H2O, 0.1 ppm H3BO3, 0.025 ppm CuSO4.5H2O, 1 ppm MnSO4.H2O and 0.05 ppm Na2MoO4.2H2O.

GROWTH CONDITIONS
- Photoperiod: 16 h light / 8 h dark
- Day/night temperatures: 25-30°C day / 15-20°C night

Any other information on materials and methods incl. tables

Each beaker was covered by a plastic sheet with three holes on the top. Three bundles of seedlings, each containing three seedlings, were cultured in the solutions through the three holes, respectively. The open areas between plastic and seedlings were sealed with sponge. Roots of the seedlings were submerged into the nutrient solution. The seedlings were allowed to grow in the nutrient solution for1week before the phytotoxicity study. Phytotoxicity experiments included three treatments. Treatment 1 was set as control without ZnO nanoparticles or Zn2+ ions added into the nutrient solution. Treatment 2 was set to study the phytotoxicity of ZnO nanoparticles which were added into the nutrient solution followed by water bath ultrasonic treatment (25°C, 100W, 40 kHz) for 1h before being planted. Treatment 3 was set to study the phytotoxicity of Zn2+ ions obtained by dissolving ZnSO4.7H2O into the nutrient solution.Allbeakers of the three treatments were randomly placed together in a growth chamber. The suspensions were stirred with a glass rod three times per day with an 8 h interval. At the end of the experiment, the seedlings were washed with flowing tap water ca. 1min. followed by rinsing with deionised water three times. Shoots and roots were separated, and their biomass was measured after drying at 70°C for 24 h. Zn concentrations in the supernatants of the hydroponic solution in treatment 2 after centrifugation (3000g for 1 h) were determined by an inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin-Elmer, Optima 4300DV, USA) at 206.2 nm wavelength. Zn contents in the shoots and roots were also measured by the ICP-OES after HNO3 digestion.
TEM, SEM and light microscopy was done following standard preparation techniques.

Results and discussions

Effect concentrations

Species	Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
Lolium perenne	12 d	other: IC50	64 mg/L	nominal	test mat.	growth	
Lolium perenne	12 d	other: IC50	51 mg/L	nominal	element (Zn)	growth	

Any other information on results incl. tables

The toxicity of Zn0 nanoparticles and Zn2+ ions to the ryegrass seedlings were evident and increased with increasing concentration of both ZnO nanoparticles and Zn2+, which could be easily observed by visual examination. The growth of seedlings in both treatments, especially with concentrations higher than 50 mg/L, was retarded with shorter roots and shoots than the control. Toxic symptoms seem more severe with Zn2+ than ZnO nanoparticles, in that shoots became yellow with concentrations of Zn2+ higher than 50 mg/L and almost withered to death at a concentration up to 1000 mg/L. As regards the dose-response curves of ZnO nanoparticles and Zn2+, there appeared a concentration threshold of both treatments, below which no significant toxic symptoms were observed. However, the seedling biomass decreased with increasing dose after the threshold. The threshold of Zn2+ for both shoot and root was ca. 20 mg/L, whereas it was around 10 and 50 mg/L of ZnO nanoparticles for ryegrass shoots and roots, respectively. The IC100 for ZnO and Zn2+ is arbitrarily taken to be near 200 mg/L, after which biomass kept nearly unchanged with further increasing concentrations. The 50% biomass inhibitory concentration (IC50) was defined in as the concentration at which the biomass equals the mean biomasses of blank and at IC100. IC50 of Zn2+ was estimated to be ca. 38 mg/L, which was lower than that of ZnO nanoparticles (ca. 64 ZnO mg/L, 51 mg Zn/L). Toxic symptoms were further examined by LM of longitudinally sectioned primary root tips. Controls showed normal development with dividing cells. However, shrank morphology of the root tips indicates severe impact of ZnO nanoparticles and Zn2+ ions. In the presence of 1000 mg/L ZnO nanoparticles or Zn2+, the epidermis and root cab were broken, the cortical cells were highly vacuolated and collapsed, and the vascular cylinder also shrank. No living cells in the root tips could be observed in the presence of 1000 mg/L Zn2+, whereas part of the vascular cells seems still alive with 1000 mg/L ZnO nanoparticles, though not active as the control. Both treatments increased total Zn in ryegrass tissues, but with different trends. There was no significant difference of root Zn contents between the two treatments with concentrations lower than 100 mg/L. When the concentrations of ZnO nanoparticles and Zn2+ ions in the nutrient solution were higher than 100 mg/L, root Zn content reducedwithincreasing Zn2+ concentration, however, increased with increasing ZnO concentration. Root Zn content in the presence of 1000 mg/L ZnO nanoparticles was 3.6 times higher than that of the 1000 mg/L Zn2+. Shoot Zn contents remained low under the ZnO nanoparticle treatments (0.25-1.36 mg/kg), and were much lower than that under die Zn2+ treatments (0.25-19.1 mg/kg). Translocation factor (TF)of Zn, defined as Zn content ratio of shoot to root, were very low (0.02-0.01) under the ZnO nanoparticle treatments, showing a decreasing tendency with increasing concentration of ZnO. However, under the Zn2+ treatments, TF (0.03-0.50) increased with increasing concentration of Zn2+. A much (1.4-50 times) lower Zn TF under the ZnO treatments than Zn2+ treatments indicates that the increasing root Zn under the ZnO treatments could be mainly from the increasing adsorption and uptake of ZnO nanoparticles by the ryegrass roots and little ZnO nanoparticles could (if any) be transported to the shoots. SEM images showed that root surface in the control and Zn2+ treatments were free of particle adherence. However, adsorption of ZnO nanoparticles and their aggregations on the root surface was evident and the coverage increased with increasing ZnO dose. The particles were observed filled in the epidermal crypt or adhered onto the surface. TEM images of the cross sections of the ryegrass roots show the presence of dark dots (particles) in the endodermis and vascular cylinder under the ZnO treatments.The dark dots distributed in the apoplast, cytoplasm, and even nuclei. One or several nanoparticles could be identified in the dark dots

covered by cytoplast as shown by higher magnification TEM image. The size of these nanoparticles were measured to be 19 ± 6nm (n = 89), identical to the size of ZnO nanoparticles. Such dark dots (i.e., particles) were not observed under either the control or Zn2+ treatments. Thus, it was concluded that the ZnO nanoparticles could enter into the endodermis and vascular cylinder of the ryegrass roots. In summary the authors concluded, ZnO nanoparticles were found able to concentrate in the rhizosphere, enter the root cells, and inhibit seedling growth; the phytotoxicity of ZnO nanoparticles could not primarily come from their dissolution in the bulk nutrient solution or the rhizosphere.

Applicant's summary and conclusion

Conclusions

In conclusion, ZnO nanoparticles seemed to lead to retarded growth. However, the validity of the observed effects are considered questionable as apparently Zn ion effects were observed and the occurrence of agglomerates were not addressed.

Executive summary

The toxicity and uptake of ZnO nanoparticles (particle size 20 nm, purity 99.5%) was studied in Lolium perenne under non-GLP/guidelien conform conditions (Lin and Xing, 2008). ZnO nanoparticles were characterized by TEM and BET analysis. Phytotoxicity experiment included three treatments; no treatment, treatment with ZnO nanoparticles or ZnSO4 heptahydrate solution, respectively. Seedling growth in both treatments was retarded with shorter roots and shoots compared to the control. In addition the seedling biomass decreased with increasing concentrations. The nominal IC50 (12 d) of ZnO nanoparticles was 64 mg/L (corresponding to 51 mg/L Zn), which was in a similar range compared to the IC50 value of ionic Zn. Furthermore, shrank morphology of the roots tips (epidermis and rootcap were broken, cortical cells were highly vacuolated and collapsed). It was also observed that ZnO nanoparticles were able to concentrate in the rhizosphere and enter the root cells. The authors suggested that the phytotoxicity of ZnO nanoparticles could not primarily come from their dissolution.

Endpoint study record: WoE.Nano.Lin and Xing (2007), radish, rape, ryegrass, lettuce, corn, cucumber

Administrative Data

Purpose flag	weight of evidence		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	This study did not follow an OECD guideline and was not performed under GLP conditions. Validity of the observed effects are questionable. NON-OECD material. TEM analysis was not performed. Nanoparticle characterization has been partially taken from the manufacturer.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Lin, D. and Xing, B.	2007	Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth	Environmental Pollution 150: 243-250					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Effects of five types of nanoparticles including ZnO (other: multi-walled carbon nanotube, aluminum, alumina, zinc) on seed germination and root growth of six higher plant species (radish, rape, ryegrass, lettuce, corn, and cucumber) were investigated after treatment with suspensions of nanoparticles.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5
common name	ZnO nanoparticles

Details on test material

- Name of test material: ZnO nanoparticles
- Physical state: solid, 20 ± 5 nm diameter- Analytical purity: > 99.5
- surface area: 50 m2/g (provided by producer); 58 m2/g (own lab)

Reference material:ZnSO4 heptahydrate

Details on analytical methods

The nanoparticles were suspended directly in deionized water (DI-water) and dispersed by ultrasonic vibration (100 W, 40 kHz) for 30 min. Small magnetic bars were placed in the suspensions for stirring before use to avoid aggregation of the particles. Zinc ion (Zn2+) solution was prepared by dissolvingZnSO4•7H2O in DI-water (El-Ghamery et al., 2003). Zinc concentration in supernatants of the nanoparticle suspensions after centrifugation (3000 g for 1 h) and filtration (0.7 mm glass filter) was determined by an inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkineElmer, Optima4300 DV, USA) at 206.2 nm wavelength. A drop of the supernatants was airdried onto a muscovite sheet (Electron Microscopy Sciences, USA), and thenwas observed by atomic force microscopy (TM-AFM, Dimension™ 3100).

Details on test substrate

- Method of application to filter paper (if used): 5 ml of test medium added to filter paper in Petri dish (100 mm x 15 mm)

Test organisms

Test organisms

Species	Brassica napus
Plant group	Dicotyledonae (dicots)
Details on test organisms	- Common name: rape- Plant family: Brassicaceae- Source of seed: Chas. C. Hart Seed Co., USA

Species	Raphanus sativus
Plant group	Dicotyledonae (dicots)
Details on test organisms	- Common name: radish- Plant family: Brassicaceae- Source of seed: Chas. C. Hart Seed Co., USA

Species	Lolium perenne
Plant group	Monocotyledonae (monocots)
Details on test organisms	- Common name: ryegrass- Plant family: Poaceae- Source of seed: Chas. C. Hart Seed Co., USA

Species	Lactuca sativa
Plant group	Dicotyledonae (dicots)
Details on test organisms	- Common name: lettuce- Plant family: Asteraceae- Source of seed: Chas. C. Hart Seed Co., USA

Species	Zea mays
Plant group	Monocotyledonae (monocots)
Details on test organisms	- Common name: corn- Plant family: Poaceae- Source of seed: Chas. C. Hart Seed Co., USA

Species	Cucumis sativus
Plant group	Dicotyledonae (dicots)
Details on test organisms	- Common name: cucumber- Plant family: Cucurbitaceae- Source of seed: Chas. C. Hart Seed Co., USA

Study design

Study type

laboratory study

Test duration type

short-term toxicity

Test type

seed germination/root elongation toxicity test

Substrate type

filter paper

Limit test

no

Total exposure duration

5 d

Test conditions

Test temperature

room temperature

pH

6.5 - 7.5

Nominal and measured concentrations

control, 20, 200, 2000 mg/L (nominal)

Details on test conditions

TEST SYSTEM
- Test container (type, material, size): Petri dish (100 mm x 15 mm)
- No. of seeds per container: 10 seeds per dish with 1 cm distance
- No. of replicates per treatment group: 3

GROWTH CONDITIONS
- Photoperiod: dark

EFFECT PARAMETERS MEASURED (with observation intervals if applicable) : seed germination and root growth after 5 days

TEST CONCENTRATIONS
- Spacing factor for test concentrations: 10
- Test concentrations: 20, 200, 2000 mg/L

Any other information on materials and methods incl. tables

Germination: Seeds were immersed in a 10% sodium hypochlorite solution for 10 min to ensure surface sterility (USEPA, 1996), then, they were soaked in DI-water, nanoparticle suspensions or Zn2+ solution for about 2 h after being rinsed three times with DI-water (Kikui et al., 2005). One piece of filter paper was put into each 100 mm x15 mm Petri dish, and 5 mL of a test medium was added. Seeds were then transferred onto the filter paper, with 10 seeds per dish and 1 cm or larger distance between each seed (Yang and Watts, 2005). Petri dishes were covered and sealed with tape, and placed in an incubator. After 5 days in the dark under room temperature, more than 80% of the control seeds had germinated and developed roots that were at least 20 mm long. Then, the germination was halted, seed germination rate was calculated, and seedling root length was measured.

Results and discussions

Effect concentrations

Species	Duration	Endpoint	Effect conc.	Nominal/Measured	Conc. based on	Basis for effect	Remarks (e.g. 95% CL)
Lactuca sativa	5 d	other: germination rate	ca. 70 %	nominal	test mat.	germination	
Lactuca sativa	5 d	NOEC	> 200 mg/L	nominal	test mat.	germination	
Brassica napus	5 d	other: IC50	ca. 20 mg/L	nominal	test mat.	other: root growth	
Raphanus sativus	5 d	other: IC50	ca. 50 mg/L	nominal	test mat.	other: root growth	
Lolium perenne	5 d	other: IC50	ca. 20 mg/L	nominal	test mat.	other: root growth	
Lactuca sativa	5 d	other: IC100	ca. 200 mg/L	nominal	test mat.	other: root growth	
Zea mays	5 d	other: IC100	ca. 200 mg/L	nominal	test mat.	other: root growth	
Cucumis sativus	5 d	other: IC100	ca. 200 mg/L	nominal	test mat.	other: root growth	

Any other information on results incl. tables

Seed germinations were not affected by the nanoparticles except for the seeds of ryegrass and corn. Seed germination ofryegrass and corn was inhibited by nano-Zn and nano-ZnO,respectively.The influence of

nanoparticle suspensions at 2000 mg/L onroot growth varied with types of nanoparticles and plant species.Phytotoxicity of nano-Zn and nano-ZnO was evident. Their suspensions significantly inhibited root growth of corn and practically terminated root development of the other five plant species whose root length at the end of experiment was unable to be accurately measured with a ruler. However, they all germinated with cotyledons sprouting out of seed coat. In this work, 1 mm was used as the minimum length to be called roots. Radish, rape and ryegrass were selected as test plant species in the following experiments.

To examine which process (seed soaking or incubation after the soaking) primarily retarded the root growth, three treatments were used: (1) both seed soaking and incubation were performed in nanoparticle suspensions; (2) seeds were soakedin nanoparticle suspensions for 2 h, and were then transferred into Petri dishes with 5 mL DI-water for incubation after being rinsed three times with DI-water; and (3) seeds were incubated in Petri dishes with 5 mL nanoparticle suspensions after being soaked in DI-water for 2 h. As described, the root growth was almost halted by seed soaking and incubation in the suspensions of nano-Zn and nano-ZnO (the first treatment). Also, root growth of radish, rape and ryegrass was nearly terminated under the third treatment (soaking in water, then incubation in suspension), while roots could grow relatively well under the second treatment (soaking in suspension, then incubation in water) though the root development of the three plant species was significantly inhibited by nano-Zn, and, that of ryegrass by nano-ZnO.

To further clarify the phytotoxicity of nano-Zn and nano-ZnO, experiments were carried out to determine the dose-response relationship of nano-Zn and nano-ZnO using the first treatment: both seed soaking and incubation in the nanoparticle suspensions or Zn2+ solutions.No significant root growth inhibition was observed under low concentrations (less than 10 mg/L for rape and ryegrass and 20 mg/L for radish). Root growth was clearly restricted with increasing concentration, and was almost terminated at 200 mg/L. Nano-Zn and nano-ZnO showed similar phytotoxicity at each of concentrations. Fifty percent root growth inhibitory concentrations (IC50) of both nano-Zn and nano-ZnO were estimated to be near 50 mg/L for radish, and near 20 mg/L for rape and ryegrass.

Two experiments were conducted to exclude Zn2+ from the phytotoxicity of nano-Zn and nano-ZnO. First, the phytotoxicity of supernatants of the nanoparticle suspensions after centrifuging at 3000 g for 1 h and filtering through 0.7 μm glass filters was measured, then the phytotoxicity of Zn2+ solutionsby dissolving ZnSO4•7H2O in DI-water was tested. No statistically significanteffect (negative or positive) was observed except for thegrowth enhancement by the supernatant of ZnO suspensionat 2000 mg/L on rape (p = 0.050) and radish (p = 0.014). Concentrations of Zn2+ in the supernatants (after centrifugationand filtration) were 0.3-3.6 mg/L. Therefore, five concentrationpoints of 0, 1, 2, 3 and 4 Zn2+ mg/L were made fromZnSO4•7H2O to investigate the phytotoxicity of Zn2+. No significant effect on the root of radish, rapeand ryegrass from these Zn2+ concentrations was observed.

Applicant's summary and conclusion

Conclusions

In conclusion, ZnO nanoparticles seemed to lead to retarded growth of terrestrial plants and decreased seed germination. However, the validity of the observed effects are considered questionable as apparently Zn ion effects were observed and the occurrence of agglomerates were not addressed.

Executive summary

Lin and Xing (2007) also investigated the effect of ZnO nanoparticles (size: 20 nm, purity > 99.5%) on seed germination and root growth under non-GLP/guideline conform conditions. Seeds were soaked in deionized water, nanoparticle suspension or ionic Zn solution for about 2 h. The results indicated that seed germination of ryegrass and corn was inhibited by ZnO nanoparticles. Additionally, ZnO nanoparticles inhibited root growth of corn and practically terminated root development of the plants used. To test the phytotoxicity of ionic Zn in the suspension of ZnO nanoparticles, their supernatant was used and the Zn2+ concentration was determined by using ICP-OES. Equivalent concentrations made from ZnSO4 heptahydrate served as reference. Since no phytotoxicity was observed, the authors assumed that the phytotoxicity of ZnO nanoparticles was not directly from their dissolution in

bulk aqueous solutions.

Endpoint study record: Disregarded.Nano.Lin and Xing (2008), ryegrass growth

Administrative Data

Purpose flag	disregarded study; robust study summary		
Study result type	experimental result		
Reliability	4 (not assignable)		
Rationale for reliability incl. deficiencies	Extended abstract. Insufficient for assessment due to missing information.		

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Lin, D. and Xing, B.	2008	Phytotoxicity of ZnO nanoparticle: inhibition of ryegrass growth	American Chemical Society, Preprints of Extended Abstracts 48(1): 276-280					

Data access

data published

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

Principles of method if other than guideline

Seeds of Lolium perenne were germinated for about two weeks after sterilisation and soaking. Seedlings were then selected and transplanted to 1000 mL beakers containing nutrient solutions and different concentrations of nano-ZnO or Zn2+. Biomass of shoots and roots were measured as well as the respective Zn-concentrations.

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	1314-13-2
EC number	215-222-5

Details on test material

- Name of test material (as cited in study report): ZnO nanoparticle (nano-ZnO)
- Physical state: solid, 20 ± 5 nm

- Analytical purity: 99.5%

Details on test substrate

Compositions of the nutrient solution were as follows: 20 ppm (NH4)2SO4, 10 ppm NH4NO3, 3.1 ppm NaH2PO4, 40 ppm K2SO4, 15 ppm CaCl2.2H2O, 0.35 ppm EDTA.FeNa.3H2O, 25 ppm MgSO4.3H2O, 20 ppm Al2(SO4)3.18H2O, 0.1 ppm ZnSO4.7H2O, 0.1 ppm H3BO3, 0.025 ppm CuSO4.5H2O, 1 ppm MnSO4.H2O and 0.05 ppm Na2MoO4.2H2O. All of these compositions were analytical grade with a purity of > 98%. The pH value of the nutrient solution wasadjusted to 7.

Test organisms

Test organisms

Species	Lolium perenne
Plant group	Monocotyledonae (monocots)
Details on test organisms	- Common name: ryegrass - Plant family: Poaceae

Study design

Study type

laboratory study

Test duration type

short-term toxicity

Test type

early seedling growth toxicity test

Substrate type

other: nutrient solution

Limit test

no

Total exposure duration

12 d

Test conditions

Test temperature

25-30°C in daytime (16 h), 15-20°C in nighttime (8 h)

pH

7

Nominal and measured concentrations

10, 20, 50, 100, 200 and 1000 mg/L (nominal), control

Details on test conditions

TEST SYSTEM
- Testing facility:
- Test container (type, material, size): 1000 mL beakers
- No. of seeds per container: 9
- No. of replicates per treatment group: 3

NUTRIENT MEDIUM (if used)
- Description: 20 ppm (NH4)2SO4, 10 ppm NH4NO3, 3.1 ppm NaH2PO4, 40 ppm K2SO4, 15 ppm CaCl2.2H2O, 0.35 ppm EDTA.FeNa.3H2O, 25 ppm MgSO4.3H2O, 20 ppm Al2(SO4)3.18H2O, 0.1 ppm ZnSO4.7H2O, 0.1 ppm H3BO3, 0.025 ppm CuSO4.5H2O, 1 ppm MnSO4.H2O and 0.05 ppm Na2MoO4.2H2O.

GROWTH CONDITIONS
- Photoperiod: 16 h light / 8 h dark
- Day/night temperatures: 25-30°C / 15-20°C

Any other information on materials and methods incl. tables

The beaker walls were wrapped with black paper to keep out light. Each beaker was covered by a plastic sheet with three holes. Three bundles of seedlings, each containing three seedlings, were cultured in the solutions through the three holes, respectively. The open areas between plastic and seedlings were sealed with sponge. Roots of the seedlings were immerged into the solutions. The seedlings were allowed to grow in the nutrient solution for one week before being used for the phytotoxicity study. The experiment included three treatments. Treatment 1 was set as control without nano-ZnO or Zn2+. Treatment 2 was set to study the phytotoxicity of nano-ZnO. The nanoparticle was added into the nutrient solution followed by sonication for 1 h before being planted. Treatment 3 was set to study the phytotoxicity of Zn2+, obtained by dissolving ZnSO4.7H2O into the nutrient solution. All beakers of the three treatments were randomly placed together in a growth chamber. During the experiment, air was continually pumped into the beakers to grow plant and to suspend particles, and the suspensions were further stirred with glass rod twice per day. At the end of the experiment, the seedlings were thoroughly washed with water and deionized water. Shoots and roots were separated and their biomasses were measured after drying at 70 ºC for 24 h. Zn ion concentrations in the supernatants of hydroponic solutions in treatment 2 after centrifugation (3,000 g for 1 h) were determined by an inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer, Optima 4300DV, USA) at 206.2 nm wavelength10. Zn contents in the shoots and roots were also measured by the ICP-OES after the HNO3 digestion and filtration (0.2 μm PTFE filter). Fresh ryegrass root was thoroughly washed and kept in a desiccator overnight. The second 2 cm rootlet was cut and coated with gold for 60 seconds, then observed by SEM (JEOL 6320FXV, Japan).

Results and discussions

Any other information on results incl. tables

The growth of seedlings was greatly inhibited under the nano-ZnO treatments. Ryegrass shoot biomass significantly (p0.05) reduced with increasing concentration of nano-ZnO in the nutrient solution, especially at the nano-ZnO concentrations higher than 20 mg/L. The shoot growth inhibition by the nano-ZnO seems the worst at 200 mg/L, with nearly constant biomass at concentrations up to 1,000 mg/L. Biomass reduction of ryegrass root showed a similar tendency. But significant root inhibition was observed only at the nano-ZnO concentrations of 100-1,000 mg/L and the root biomass kept nearly unchanged in this concentration range.No significant difference of the ryegrass biomass between the treatments of Zn2+ andnano-ZnO could be observed. But the yellow and withered shoots at higherZn2+ concentrations indicates that Zn2+ might be more toxic tothe ryegrass than nano-ZnO. Hence, one may wonder if the phytotoxicity of nano-ZnOcame from the dissolution of nano-ZnO. Zinc ion concentrations in the nano-ZnOtreated nutrient solutions after centrifugation and filtration were lower than 6 mg/L. Nosignificant toxicity of Zn2+at 6 mg/L was observed to the ryegrass. Thus, thephytotoxicity of nano-ZnO could not result from its direct dissolution in the nutrientsolution whose pH was adjusted to be 7.Both treatments increased total Zn in the ryegrass tissues, but with different trends. There was no significant difference of root Zn contents between thetreatments of nano-ZnO and Zn2+ with concentrations lower than 100 mg/L. When theconcentrations of nano-ZnO or Zn2+in the nutrient solution were higher than 100 mg/L,root Zn content reduced with increasing Zn2+ concentration; however, increased withinexperimental concentrations of nano-ZnO. The reduction of root Zn content at higherZn2+concentrations may be due to the great inhibition of plant activity while theincreasing root Zn under the nano-ZnO treatments could be from increasing adsorptionof nano-ZnO by ryegrass root. It was evident that the nano-ZnO coverage on rootsurface increased with increasing nano-ZnO concentration in

the nutrient solution. Shoot Zn contents (0.25-1.36 mg/kg) remained low under the nano-ZnOtreatments and were much lower than that under the Zn2+ treatments (0.25-19.1 mg/kg).This indicates that ZnO particles might not directly transport from root to shoot, ordissolve to be Zn2+, at least not much, at root surface or inside root tissue. Therefore it was concluded,the phytotoxicity of nano-ZnO could not mainly come from its dissolution at root surfaceor inside root tissue.