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

DOSSIER ON NANOCCLAYS

**Series on the Safety of Manufactured Nanomaterials
No. 47**

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ENV/JM/MONO(2015)10
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OECD Environment, Health and Safety Publications

Series on the Safety of Manufactured Nanomaterials

No. 47

DOSSIER ON NANOCCLAYS



INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

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

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No. 51, *Dossier on Silicon dioxide (2015)*

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

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 testing². This list comprised: i) fullerenes (C60); ii) single-walled carbon nanotubes (SWCNTs); iii) multi-walled carbon nanotubes (MWCNTs); iv) silver nanoparticles; v) titanium dioxide; vi) cerium oxide; vii) zinc oxide; viii) silicon dioxide; ix) dendrimers; x) nanoclays; and xi) gold nanoparticles. One fundamental criterion for selecting these materials was that they should be either in commercial use at the time or expected to be in the near future. At the same time, other considerations were also given attention, such as the production volume of the materials, the likely availability of such materials for testing and the existing information that would readily be available on the materials.

It was also agreed that 59 endpoints would be addressed³ for each material corresponding to the following categories: i) nanomaterial information/ identification; ii) physical-chemical properties and material characterisation; iii) environmental fate; iv) toxicological and eco-toxicological effects; v) environmental toxicology; vi) mammalian toxicology; and vii) material safety. These endpoints were judged to be most important based largely on the general experience of testing chemicals, while taking into account the potentially different or new properties of nanomaterials. It is worth noticing that it was not expected that testing for all of the listed endpoints would be necessary for each of the selected MNs.

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

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

<http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforhetestingofchemicals.htm>

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

³ As specified in the Guidance Manual, "address" includes the term "completed" which provides that all dossiers will contain the identified endpoint information. Note that for some endpoints (for example, solubility) it is specified that the endpoint must be "completed". In such instances "completed" means that all Dossiers will be providing this endpoint information.

[ENV/JM/MONO(2009)20/REV]⁴. The objective of this Guidance Manual was to guide sponsors⁵ in the testing of the materials while ensuring that the information collected was reliable, accurate, consistent and therefore also comparable. The Guidance Manual addressed a whole range of issues including the organisation of the work.

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

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

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

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

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)

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 Nanoclays Bentonite. 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 IUCLID⁹ 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 the Nanoclays Bentonite. 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 participating stakeholders from Denmark, the United States, and the European Commission.

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 OECD (BIAC) for leading the Testing Programme for Nanoclays. 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 Denmark, the United States, and the European Commission.

TABLE OF CONTENTS

PREAMBLE.....	7
FOREWORD.....	9
ACKNOWLEDGMENTS.....	10
1. GENERAL INFORMATION	14
1.1 Identification.....	14
1.2 Composition.....	15
1.3 Identifiers	15
1.4 Analytical information	15
1.5 Joint submission.....	15
1.6 Sponsors.....	15
1.7 Suppliers	15
1.8 Recipients.....	15
1.9 Product and process oriented research and development.....	15
2. CLASSIFICATION & LABELLING AND PBT ASSESSMENT.....	15
2.1 GHS	15
2.2 DSD – DPD	15
3. MANUFACTURE, USE AND EXPOSURE.....	16
3.1 Technological process.....	16
3.2 Estimated quantities.....	16
3.3 Sites.....	16
3.4 Information on mixtures	16
3.5 Life Cycle description.....	16
3.6 Uses advised against	16
3.7 Exposure Scenarios, exposure and risk assessment	16
3.8 Biocidal information	16
3.9 Emissions Information.....	16
3.10 Application for authorisation of uses.....	16
4. PHYSICAL AND CHEMICAL PROPERTIES.....	16
4.1 Stability and homogeneity	16
4.2 Appearance/physical state/colour	18
4.3 Melting point/freezing point	18
4.4 Boiling point	18
4.5 Density	18
4.6 Particle size distribution (Granulometry).....	18
4.7 Vapour pressure	26
4.8 Partition coefficient.....	26
4.9 Water solubility.....	26

4.10 Solubility in organic solvents / fat solubility	26
4.11 Surface tension.....	26
4.12 Flash point.....	26
4.13 Auto flammability	26
4.14 Flammability	26
4.15 Explosiveness.....	26
4.16 Oxidising properties.....	26
4.17 Oxidation reduction potential	26
4.18 Stability in organic solvents and identity of relevant degradation products	26
4.19 Storage stability and reactivity towards container material	26
4.20 Stability: thermal, sunlight, metals	26
4.21 pH.....	26
4.22 Dissociation constant	26
4.23 Viscosity	26
4.24 Additional physico-chemical information	26
4.25 Agglomeration/aggregation	27
4.26 Crystalline phase.....	29
4.27 Crystallite and grain size.....	29
4.28 Aspect ratio/shape.....	29
4.29 Specific surface area	29
4.30 Zeta potential	31
4.31 Surface chemistry.....	31
4.32 Dustiness.....	34
4.33 Porosity	34
4.34 Pour density	34
4.35 Photocatalytic activity.....	34
4.36 Radical formation potential.....	34
4.37 Catalytic activity	34
5. ENVIRONMENTAL FATE AND PATHWAYS.....	34
6. ECOTOXICOLOGICAL INFORMATION	34
6.1 Aquatic toxicity.....	34
6.1.1 Short-term toxicity to fish	34
6.1.2 Long-term toxicity to fish.....	39
6.1.3 Short-term toxicity to aquatic invertebrates	39
6.1.4 Long-term toxicity to aquatic invertebrates	43
6.1.5 Toxicity to aquatic algae and cyanobacteria	43
6.1.6 Toxicity to aquatic plants other than algae.....	49
6.1.7 Toxicity to microorganisms.....	49
6.1.8 Toxicity to other aquatic organisms	49
6.2 Sediment toxicity	49
6.3 Terrestrial toxicity.....	55
6.4 Biological effects monitoring	55
6.5 Biotransformation and kinetics	55

6.6 Additional ecotoxicological information	55
7. TOXICOLOGICAL INFORMATION	55
8. ANALYTICAL METHODS	55
9. RESIDUES IN FOOD AND FEEDINGSTUFFS	55
10. EFFECTIVENESS AGAINST TARGET ORGANISMS	55
11. GUIDANCE ON SAFE USE	55
12. LITERATURE SEARCH.....	55
13. ASSESSMENT REPORTS	55
14. INFORMATION REQUIREMENTS	55
APPENDIX	56

1. GENERAL INFORMATION

1.1 Identification

Reference substance:

Bentonite

EC number:

215-108-5

EC name:

Bentonite

CAS number:

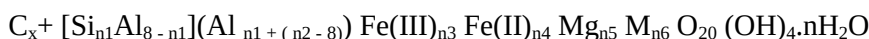
1302-78-9

Type of substance:

Existing chemical

Structural formula/molecular structure:

Hydrated Sodium Calcium Aluminium Magnesium Silicate Hydroxide



C represents cations that compensate the negative structural charge and are usually Ca^{2+} , Mg^{2+} , Na^+ , with charge x^+ lying between 0.5-1.2

n_i , $i=1,2,3,4,5,6$ are the stoichiometric coefficients associated with the structural cations

For dioctahedral clays with divalent M the sum of n 's =12

For trioctahedral clays with monovalent M the sum of n 's =14

Composition of nanomaterial being tested (including degree of purity, known impurities or additives):

Bentonite may contain crystalline silica in quantity up to 3 %.

Basic morphology

Dry, fine powder

Trade names:

Bentonite Accofloc352 AccugelF AEG325 Akajo Aquagel Askangel Baroco Benclay Bengel BengelFW BentoGrout BentoliteL Bentone660 BentonilC BentonitT Bentonite,calcian-sodian Name Bentosolon82 BlackHilsBH200 BulgarbenBA Cosintam403 Culvin CVIS DetercolP2 Filgel Fulbent570 GK129 GK129SA3 GK129SA5 Gumbrin Hi-Jel1 HPM20 InviteFGB K306 KA3 KA3(clay) KorthixH-NF Kunigel Kunigel3V KunigelVA KunigelVI KunigelVO LaundrosilDG LaviosaAGB LD16 MineralColoid101 MineralColoid103 Nalco650 NewDOrben OptigelCK OptigelWX Organosorb Otaylite S-Ben Steecoben Steecomul Tone Tone(clay) Tyotix55M Ultrogel300 UltrogelAcAA Veegum HS Volclay VolclayBC VolclayHPM VolclayHPM20 VolclayKWK VolclayNF-BC VolclaySPV200 WesternBond Wilkonite YelowStone

1.2 Composition

1.3 Identifiers

1.4 Analytical information

1.5 Joint submission

1.6 Sponsors

BIAC (Nanotechnology Industry Association)

1.7 Suppliers

1.8 Recipients

1.9 Product and process oriented research and development

2. CLASSIFICATION & LABELLING AND PBT ASSESSMENT

2.1 GHS

2.2 DSD – DPD

Classification and Labelling according 67/548/EEC (DSD):
No classification/labeling required (no dangerous properties)
Not classified

3. MANUFACTURE, USE AND EXPOSURE

3.1 Technological process

3.2 Estimated quantities

3.3 Sites

3.4 Information on mixtures

3.5 Life Cycle description

3.6 Uses advised against

3.7 Exposure Scenarios, exposure and risk assessment

3.8 Biocidal information

3.9 Emissions Information

3.10 Application for authorisation of uses

4. PHYSICAL AND CHEMICAL PROPERTIES

4.1 Stability and homogeneity

Endpoint study record: 2011-01-17 NPL_Stability monitoring_Turbidity_DI Water

Administrative Data

Purpose flag

Study result type

Experimental results

Study period

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

Data source

Reference

Data access

Data submitter is data owner.

Data protection claimed

Yes, but willing to share

Materials and methods**Test guideline****Methods**

Other. Turbidity

Principles of method if other than guideline

Turbidity was measured using HF Scientific–Micro100 RI turbidity meter (Cole-Palmer,UK); this meter has an infrared light source that meets the international standard ISO 7027 for turbidity measurements. The meter was calibrated on standards, which are based on AMCO-AEPA-1 micro spheres; these standards are traceable to standard formazin suspension. Standard values of 1000, 10 and 0.02 NTU were used to calibrate the meter. Prior to use, the meter was allowed to warm up for 30 minutes. Sample cuvetes (HF Scientific (USA) were used to hold the sample. Note that glass thickness may vary from cuvette to cuvette and within the same cuvette. Hence, individual vials were indexed; indexing of the cuvette entails finding the point of the cuvette that light passes through that gives the lowest reading; once indexed the holder can be marked accordingly. Prior to their use, cuvettes were cleaned, in accordance to manufacturer's instructions. This involved washing the interior and exterior of the cuvette with a detergent (2% Hellmanex in DI water); it was then rinsed several times in distilled water before finally rinsing in DI water. The cuvette was further rinsed with the sample two times before filling (30ml) and analysed. The cuvette was placed into the meter and signal allowed to settle before taking readings. Prior to testing the slurries were diluted in the corresponding blank media; a ratio of 1:10 of slurry: blank media. The measurement signal was compared with corresponding slurries with no dilution step involved. It was observed that this dilution step was required in order to avoid saturation of the turbidity signal. Air bubbles from the samples were removed by placing the sample under vacuum for several hours. Turbidity readings were taken at regular time intervals.

Instrument

HF Scientific – Micro100 RI turbidity meter (Cole-Palmer, UK)

Calibration

The meter was calibrated on standards, which are based on AMCO-AEPA-1 microspheres; these standards are traceable to standard formazin suspension. Standard values of 1000, 10 and 0.02 NTU were used to calibrate the meter

GLP compliance**Test materials**

Test material equivalent to submission substance identity.

Sample identification number: 0053

Test material form

Dispersion

Details on test material**Confidential details on test material**

Any other information on materials and methods incl. tables**Results and discussions****Overall remarks, attachments**

Results show that NM-600 Bentonite in DI water, as dispersed using our in-house dispersion protocol with a 20 s exposure time, has a much smaller half life value when compared to Tetra Pak's G1 sample. This suggests that this dispersion is less stable and thus sediments out at a faster rate. However, when total sonication exposure time in our dispersion was increased from 20 s to 460 s, this had a dramatic effect on the corresponding half-life value, in that the dispersion became less prone to sedimentation. In fact, the 460 s exposure resulted in a much larger half life value compared to Tetra Pak's G1 sample; this is indicative of the fact that our in-house protocol with 460 s ultrasonic exposure time had resulted in a more stable dispersion than the corresponding Tetra Pak's G1 sample. Certainly, results indicate that exposure time governs the overall stability of the dispersion. It is likely therefore that increasing the exposure time can result in increasing dissolution process of the clay in the DI water; this can potentially change the viscosity of the dispersion i.e. making it more viscous. The more viscous the solution the less prone the dispersion will be to sedimentation.

Remarks on results including tables and figures**Attached background material**

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf / 5.9 MB (application/pdf): Appendix

Applicant's summary and conclusion**Conclusions****Executive summary****Cross-reference to other study****4.2 Appearance/physical state/colour****4.3 Melting point/freezing point****4.4 Boiling point****4.5 Density****4.6 Particle size distribution (Granulometry)**

Endpoint study record: 2011-01-17 NPL_Stability monitoring_Turbidity_DI Water**Administrative Data****Purpose flag****Study result type**

Experimental results

Study period

Reliability

2 (reliable with restriction)

Rationale for reliability incl. deficiencies**Data source****Reference****Data access**

Data submitter is data owner.

Data protection claimed

Yes, but willing to share

Cross-reference to same study**Materials and methods****Test guideline****Methods**

SEM

Details on methods and data evaluation

SEM images were obtained using a Supra 40 field emission scanning electron microscope from Carl Zeiss (Welwyn Garden City, Hertfordshire, UK), in which the optimal spatial resolution of the microscope was a few nanometres. In-lens detector images were acquired at an accelerating voltage of 15 kV, a working distance of ~3 mm, and a tilt angle 0°. For analysis of the “as received” nanoparticle powder, a sample of the powder was sprinkled over a SEM carbon adhesive disc; one side of the carbon disc was placed securely on a metal stub, whilst the other side was exposed to the nanoparticle powder. Excess powder was removed by gently tapping the stub on its side until a light coating of powder on the surface became apparent. Samples were thinly sputtered with gold using an Edwards S150B sputter coater unit (BOC Edwards, UK). Sputtering was conducted under vacuum (~7 mbar or ~0.7 mPa), while passing dry argon into the coating chamber. Typical plate voltage and current were 1200 V and 15 mA, respectively. The sputtering time was approximately 10s, which resulted in an estimated gold thickness of not more than 2 nanometres being deposited on top of the substrate. An adequate magnification was chosen for image acquisition e.g. for the estimation of primary particle mean diameter and the estimation of aggregates/agglomerates. The shape and limits of the primary particles and agglomerates/aggregates should become apparent. SEM micrographs were analysed by manually tracing contours of primary particles on to a transparency sheet. The transparency sheet was scanned for further image analysis using ImageJ software, which automatically calculated particle diameter dimensions.

Instrument

Carl Zeiss Supra 40 field emission scanning electron microscope

Calibration

GLP compliance

Test materials

Test material equivalent to submission substance identity.

Sample identification number: 0153

Test material form

Dry bulk

Details on test material

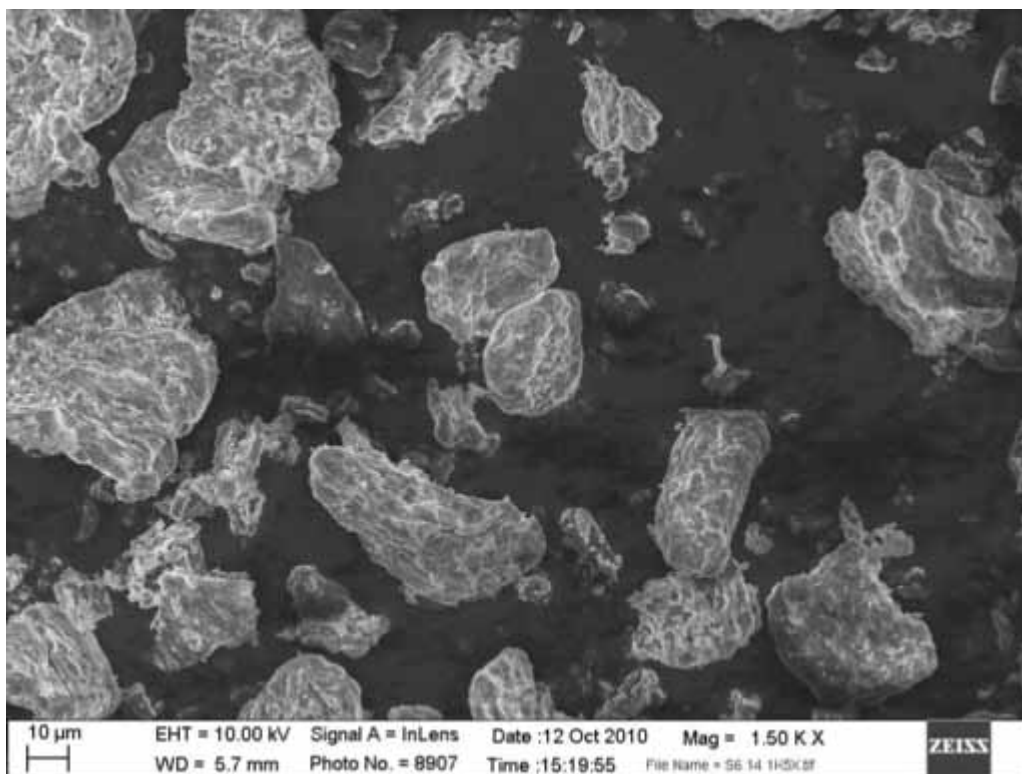
Confidential details on test material

Any other information on materials and methods incl. tables

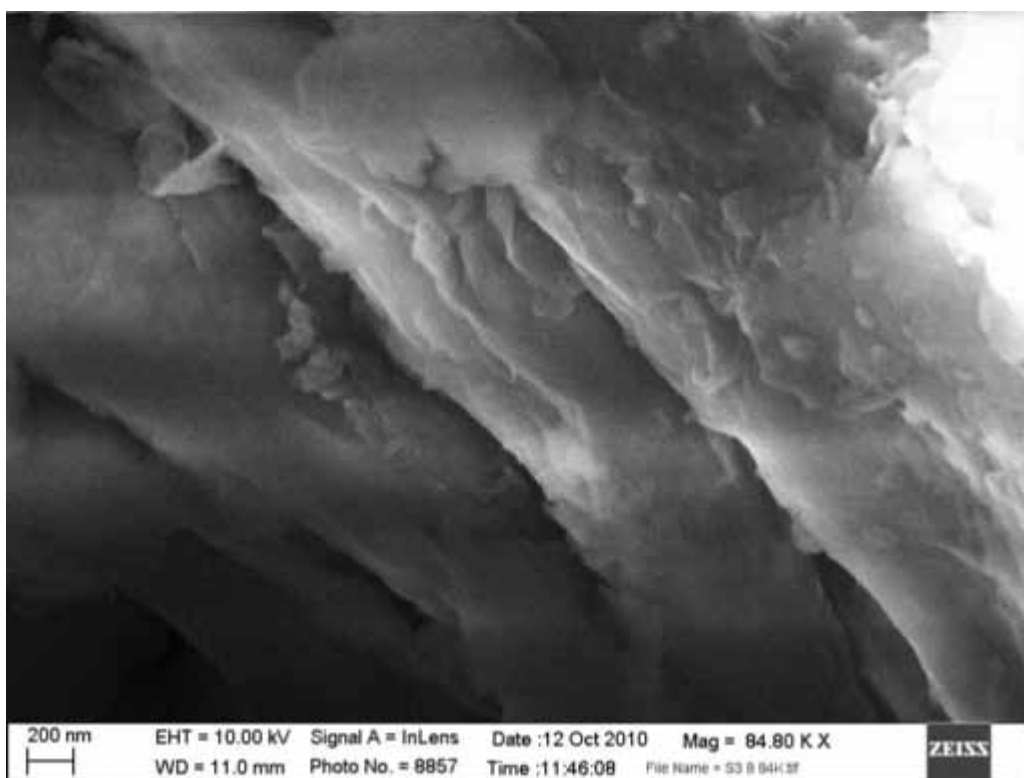
Results and discussions

Figure below shows a typical SEM image of the “as received” Bentonite clay powders at low and high magnification. Images show that:

- a) Particles are highly aggregated/agglomerated, with tens of micron size particles evident.
- b) Each particle is made up of sheet-like units, held together.



a)



b)

Figure. SEM images of NM-600

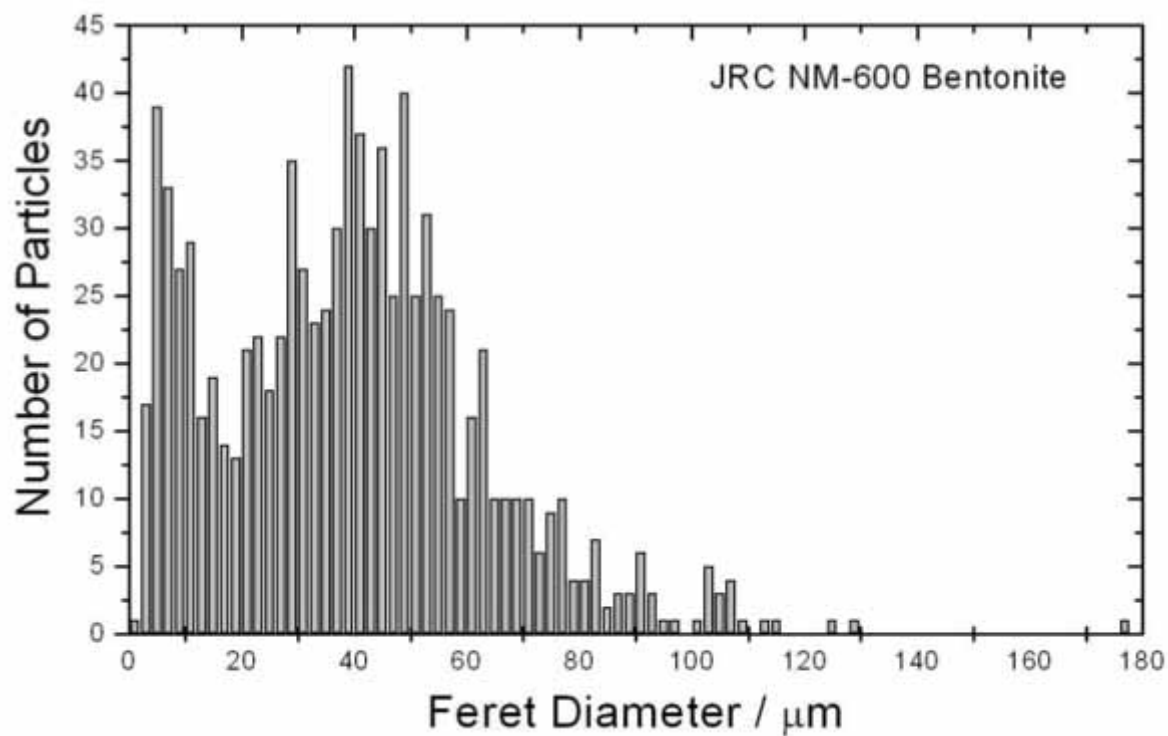


Figure. Particle size distribution of Bentonite clay NM-600 (0153), as obtained from the SEM images and analysing ~ 900 clay particles.

Mean diameter

Ca 39.5 μm (micrometer)

Remarks on results including tables and figures

Figure shows the particle size distribution of Bentonite clay NM-600 (0153), in which the clay particles are present in the form of clustered sheets of particles. The corresponding mean Feret's diameter of the particles is 39.5 microns +/- 23.8 microns; the SD value reported here gives an indication of the polydispersity. It is evident that the clay sample is highly polydispersed i.e. broad size distribution. Here, we report Feret's diameter, as this parameter is widely used for the analysis of irregular shaped particles. The Feret diameter can be defined as the "maximum calliper length" i.e. the longest distance between any two points along the selection boundary

Overall remarks, attachments

SEM image of the "as received" Bentonite clay powders at low and high magnification indicate that:

- a) Particles are highly aggregated/agglomerated, with tens of micron size particles evident.
- b) Each particle is made up of sheet-like units, held together

Remarks on results including tables and figures**Attached background material**

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf / 5.9 MB (application/pdf): Appendix

Applicant's summary and conclusion**Conclusions**

The corresponding mean Feret's diameter of the particles is 39.5 microns +/- 23.8 microns determined via SEM.

Executive summary**Cross-reference to other study*****Endpoint study record: 2011-01-06_NPL_Particle size, size distribution_CPS_DI Water*****Administrative Data****Purpose flag****Study result type**

Experimental results

Study period**Reliability**

2 (reliable with restriction)

Rationale for reliability incl. deficiencies**Data source****Reference****Data access**

Data submitter is data owner.

Data protection claimed

Yes, but willing to share

Cross-reference to same study**Materials and methods****Test guideline****Methods**

Ultracentrifuge

Details on methods and data evaluation

Particle size distribution by centrifugal sedimentation was acquired using CPS Disc Centrifuge Model DC 20000 instrument (Analytik Ltd, UK). At the start of the method, the centrifuge was brought up to speed by partially filling the disc with a sucrose gradient fluid and dodecane cap fluid. The purpose of the gradient fluid was to stabilise the sedimentation; the purpose of the cap fluid was to maintain the gradient inside the disc. The disc centrifuge was then allowed to equilibrate at 6000 rpm for 1 hour. This gradient is stable over a 6-hour period. Prior to testing, the slurries were diluted in the corresponding blank media, with a ratio of 1:10 of slurry: blank media. This dilution step was necessary to provide the optimum signal-to-noise ratio. 0.1 ml of the sample was injected into the disc; a calibration standard was injected after every three samples. Analysis was run against a calibration standard (NIST traceable standard, PVC 0.377 μm). The Disc Centrifuge Control System software (CPS Instruments Inc.) was used to acquire and process the data

Instrument

CPS Disc Centrifuge Model DC 20000 instrument (Analytik Ltd, UK)

Calibration

Analysis was run against a calibration standard, NIST traceable standard, PVC 0.377 micron.

GLP compliance**Test materials**

Test material equivalent to submission substance identity.

Sample identification number: 052

Test material form

Dispersion

Details on test material**Confidential details on test material****Any other information on materials and methods incl. tables**

Sample media: DI water

Results and discussions**Mean diameter**

288 nm

Particle size

Percentile D10: mean 631 nm

Percentile D50: mean 166 nm

Percentile D90: mean 87 nm

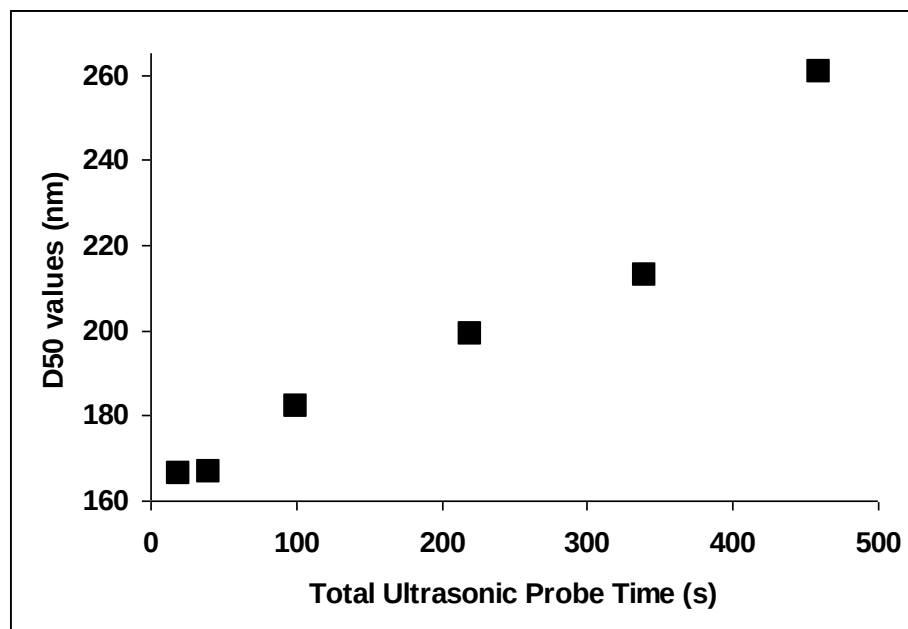
Remarks on results including tables and figures

Above results were done using sonication time of 20 seconds. Other sonication times were also performed and the results are given below.

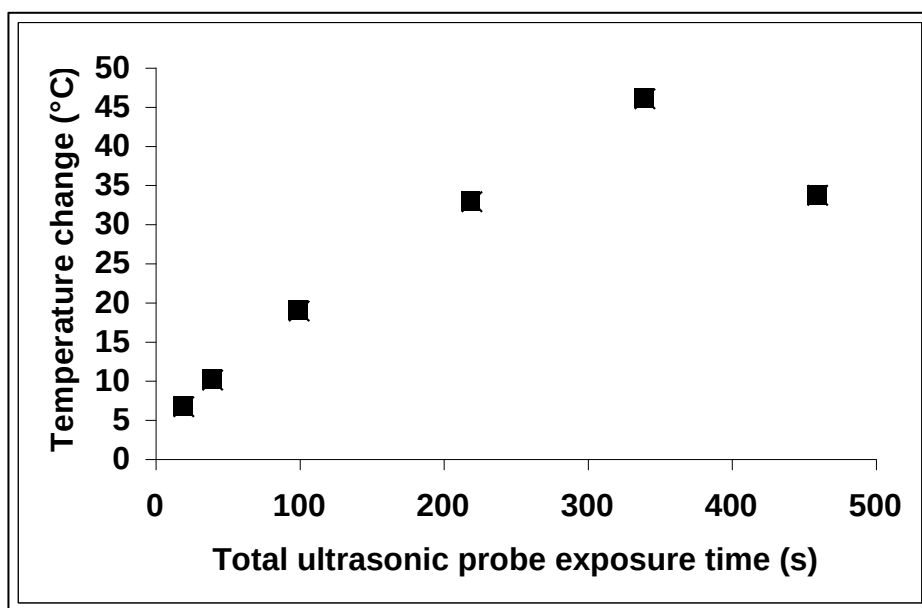
Table: CPS disc centrifugal sedimentation results showing: the equivalent mean spherical particle diameter of dispersed NM-600 BENTONITE 0052 and the corresponding D10, D50, D90 values (oversize percentiles) from the averaged CPS measurements

Total Ultrasonic Probe Exposure Time/ (s)	Mean particle size (nm)	D10 (nm)	D50 (nm)	D90 (nm)
20	288	631	166	87
40	282	646	167	83
100	383	1130	182	87
220	407	1123	199	90
340	396	1022	213	94
460	506	1320	261	93

Results indicate that as total sonication time increases, both mean particle size and the corresponding oversize percentiles (D10, D50, D90) increases. In particular, this trend is very clear when D50 values are plotted against total ultrasonic probe time, in which an almost linear relationship exists between the two variables.



a)



b)

Figure: Effects on dispersion characteristics of NM-600 (0052) BENTONITE dispersed in DI water, using the recommended dispersion protocol, as a function of total ultrasonic probe exposure time on: a) D_{50} values b) temperature change measured in the dispersion.

Results indicate that the clay aggregate/agglomerate, resulting in the formation of larger particle sizes, increases with sonication treatment time. The increase in sonication treatment time is also accompanied by an increase in the temperature of the dispersion, which is hardly surprising. The increase in temperature can promote further aggregation/agglomeration amongst the particles. A rather strange effect is observed when total exposure time is 460s, in which the temperature change in the dispersion started to decrease.

Overall remarks, attachments

Remarks on results including tables and figures

Attached background material

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf / 5.9 MB (application/pdf): Appendix

Applicant's summary and conclusion

Conclusions

Results indicate that as total sonication time increases, both mean particle size and the corresponding oversize percentiles (D_{10} , D_{50} , D_{90}) increases. In particular, this trend is very clear when D_{50} values are plotted against total ultrasonic probe time, in which an almost linear relationship exists between the two variables.

Executive summary

Cross-reference to other study

4.7 Vapour pressure

4.8 Partition coefficient

4.9 Water solubility

4.10 Solubility in organic solvents / fat solubility

4.11 Surface tension

4.12 Flash point

4.13 Auto flammability

4.14 Flammability

4.15 Explosiveness

4.16 Oxidising properties

4.17 Oxidation reduction potential

4.18 Stability in organic solvents and identity of relevant degradation products

4.19 Storage stability and reactivity towards container material

4.20 Stability: thermal, sunlight, metals

4.21 pH

4.22 Dissociation constant

4.23 Viscosity

4.24 Additional physico-chemical information

4.25 Agglomeration/aggregation

Endpoint study record: 2011-01-07_NPL_Agglomeration/aggregation_SEM

Administrative Data

Purpose flag

Study result type

Experimental results

Study period

Reliability

2 (reliable with restriction)

Rationale for reliability incl. deficiencies

Data source

Reference

Data access

Data submitter is data owner.

Data protection claimed

Yes, but willing to share

Cross-reference to same study

Materials and methods

Test guideline

Methods

SEM

Details on methods and data evaluation

SEM images were obtained using a Supra 40 field emission scanning electron microscope from Carl Zeiss (Welwyn Garden City, Hertfordshire, UK), in which the optimal spatial resolution of the microscope was a few nanometres. In-lens detector images were acquired at an accelerating voltage of 15 kV, a working distance of ~3 mm, and a tilt angle 0°. For analysis of the “as received” nanoparticle powder, a sample of the powder was sprinkled over a SEM carbon adhesive disc; one side of the carbon disc was placed securely on a metal stub, whilst the other side was exposed to the nanoparticle powder. Excess powder was removed by gently tapping the stub on its side until a light coating of powder on the surface became apparent. Samples were thinly sputtered with gold using an Edwards S150B sputter coater unit (BOC Edwards, UK). Sputtering was conducted under vacuum (~7 mbar or ~0.7 mPa), while passing dry argon into the coating chamber. Typical plate voltage and current were 1200 V and 15 mA, respectively. The sputtering time was approximately 10s, which resulted in an estimated gold thickness of not more than 2 nanometres being deposited on top of the substrate. An adequate magnification was chosen for image acquisition e.g. for the estimation of primary particle mean diameter and the estimation of aggregates/agglomerates. The shape and limits of the primary particles and agglomerates/aggregates should become apparent. SEM micrographs were analysed by manually tracing contours of primary particles on to a transparency sheet. The transparency sheet was scanned for further image analysis using ImageJ software, which automatically calculated particle diameter dimensions.

Instrument

Carl Zeiss Supra 40 field emission scanning electron microscope

Calibration**GLP compliance****Test materials**

Test material equivalent to submission substance identity.

Sample identification number: 0153

Test material form

Dry bulk

Details on test material**Confidential details on test material****Any other information on materials and methods incl. tables****Results and discussions****Mean diameter**

Ca 39.5 μm (micrometer)

Remarks on results including tables and figures

The corresponding mean Feret's diameter of the particles is 39.5 microns +/- 23.8 microns; the SD value reported here gives an indication of the polydispersity. It is evident that the clay sample is highly polydispersed i.e. broad size distribution.

Overall remarks, attachments

SEM image of the "as received" Bentonite clay powders at low and high magnification show that:

- a) Particles are highly aggregated/agglomerated, with tens of micron size particles evident.
- b) Each particle is made up of sheet-like units, held together

Remarks on results including tables and figures**Attached background material**

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf / 5.9 MB (application/pdf): Appendix

Applicant's summary and conclusion**Conclusions**

Particles are highly aggregated/agglomerated, with tens of micron size particles evident. Each particle is made up of sheet-like units, held together.

Executive summary**Cross-reference to other study**

4.26 Crystalline phase**4.27 Crystallite and grain size****4.28 Aspect ratio/shape****4.29 Specific surface area*****Endpoint study record: 2011-01-06_NPL_Specific surface area_BET*****Administrative Data****Purpose flag****Study result type**

Experimental results

Study period**Reliability**

1 (reliable without restriction)

Rationale for reliability incl. deficiencies**Data source****Reference****Data access**

Data submitter is data owner.

Data protection claimed

Yes, but willing to share

Cross-reference to same study**Materials and methods****Test guideline****Methods**

BET

Principles of method if other than guideline

BET (Brunauer, Emmett and Teller) Multipoint Specific Surface Area determination

Details on methods and data evaluation

BET surface area measurements were determined using Autosorb-1 (Quantachrome Instruments). The Autosorb-1 was calibrated using a quartz rod of a known volume, which is traceable to NIST. This calibration was then further checked using two BAM certified reference materials: BAM-PM- 102 (nominal SSA 5.41m²g⁻¹) and BAM-PM-104 (nominal SSA 79.8 m²g⁻¹). These two reference materials allowed the range of SSA of the nanoparticles to be encompassed with known specific surface area materials, thus adding confidence to the measurements. Surface area measurements were acquired using an 11-point BET gas adsorption method, with nitrogen as the adsorbate. Prior to analysis, the powdered

sample was transferred to a sample bulb, then sealed and subsequently de-gassed overnight at 300 °C under a high vacuum and subsequently weighed on a analytical balance in order to determine the sample mass after the degassing step..

Instrument

Autosorb-AS1

Calibration

Manifold calibration using a calibrated quartz rod SSA calibration carried out using BAM CRM BAM-PM-102 and BAM CRM BAM-PM-104

GLP compliance**Test materials**

Test material equivalent to submission substance identity.

Sample identification number: 073

Test material form

Dry bulk

Details on test material**Confidential details on test material****Any other information on materials and methods incl. tables****Results and discussions****Specific surface area**

Mean: 51.9 m²/g

Standard deviation: 1.6 m²/g

Remarks on results including tables and figures**Overall remarks, attachments****Remarks on results including tables and figures****Attached background material**

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf / 5.9 MB (application/pdf): Appendix

Applicant's summary and conclusion**Conclusions**

The BET result shows that the specific surface area of the Bentonite clay (NM600, 0073) was 51.9 +/- 1.6 m²/g; errors (2 SD) reported here are the uncertainty of the result when acquiring a “one sample” dataset as reported by the BET gas adsorption technique

Executive summary**Cross-reference to other study**

4.30 Zeta potential

4.31 Surface chemistry

Endpoint study record: 2011-01-07_NPL_Surface chemistry_EDX

Administrative Data

Purpose flag

Study result type

Experimental results

Study period

Reliability

2 (reliable with restriction)

Rationale for reliability incl. deficiencies

Data source

Reference

Data access

Data submitter is data owner.

Data protection claimed

Yes, but willing to share

Cross-reference to same study

Materials and methods

Test guideline

Methods

Other: Energy dispersive X-ray (EDX)

Principles of method if other than guideline

Details on methods and data evaluation

The JRC NM-600 Bentonite (Sample Identification Number = 0153) was mounted in two different ways for SEM examination:

1. Sample sprinkled as a thin layer onto an adhesive carbon disk
2. Sample packed into a specially adapted SEM stub.

Energy dispersive X-ray (EDX) microanalyses were carried out in a Camscan MX2500 SEM (serial number: 149, gun type: thermionic emission / W-filament) fitted with an Oxford Instruments Ltd. "INCA Energy+" EDX system (Si-Li EDX detector serial number: 14117 3390-339-51). The SEM/EDX conditions were set to the following standard operating conditions: 20kV accelerating voltage, 35 mm working distance, 50 s count time, 23 25 % detector dead time and process time: 5 µs. The beam current was adjusted to give approximately 3000 counts per second (cps) on a pure cobalt (Co) specimen (gain calibration). EDX spectra were acquired at various magnifications in the range: x20-x50 and scanning the electron beam in selected rectangular areas within a preselected visual field. All EDX spectra were processed using an Oxford Instruments Ltd. 'Inca Energy+' ZAF matrix correction program called XPP,

(where: Z-atomic number, A absorption and F fluorescence). In EDX microanalysis, it is not possible to determine quantitatively the concentrations of the light elements ($Z < 11$) such as C, N and O from the measured spectra with reasonable accuracy due to instrumental factors, the type of material being analyzed and inaccurate ZAF corrections. For $Z \leq 11$ (Na), the EDX detector has an approximate minimum detection limit of 0.1wt%. Hence, the concentrations were calculated using known stoichiometric combinations of the detected elements with oxygen e.g. Na₂O, CaO, Fe₂O₃ etc.

Instrument

Camscan MX2500 SEM (serial number: 149, gun type: thermionic emission / W-filament) fitted with an Oxford Instruments Ltd. "INCA Energy+" EDX system (Si-Li EDX detector serial number: 14117 3390-339-51)

Calibration

GLP compliance

Test materials

Test material equivalent to submission substance identity.

Sample identification number: 0153

Test material form

Dry bulk

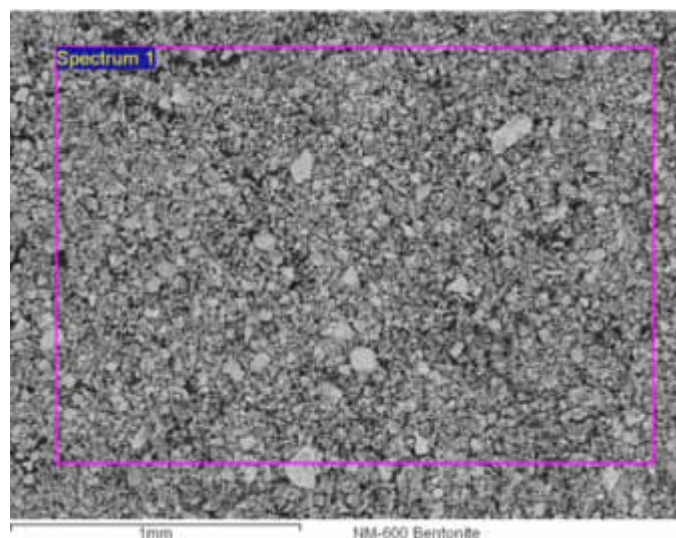
Details on test material

Confidential details on test material

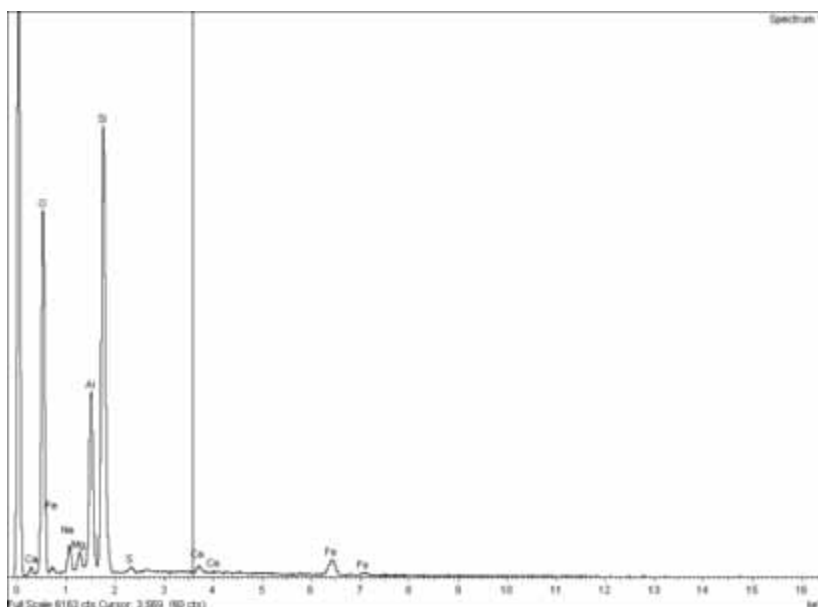
Any other information on materials and methods incl. tables

Results and discussions

Figure shows the EDX analysis of NM-600 (0153) Bentonite performed on the selected marked areas.



a)



b)

Figure. EDX analysis of NM-600 (0153) Bentonite showing typical: a) selected area for EDX analysis, as marked by the pink box. b) EDX spectrum.

Atomic composition

Table: Elemental composition (atomic %) according to EDX analysis of NM-600 (0153) Bentonite by stoichiometry (oxygen) of sample that was: a) applied as a thin layer on to an SEM stub b) packed into a specially adapted SEM stub. Table shows the average value (and corresponding 1 SD) from 12 replicate spectra.

a)	Composition / atomic%								
	Na	Mg	Al	Si	S	Ca	Fe	O	Sum
Average	2.85	1.29	8.61	22.25	0.27	0.29	1.61	62.84	100.00
SD	0.38	0.10	0.22	0.37	0.21	0.10	0.31	0.15	

b)	Composition / atomic%								
	Na	Mg	Al	Si	S	Ca	Fe	O	Sum
Average	2.79	1.30	8.51	22.16	0.39	0.37	1.57	62.90	100.00
SD	0.51	0.11	0.40	0.70	0.39	0.20	0.42	0.21	

Remarks on results including tables and figures

Overall remarks, attachments

EDX results show that the NM-600 (0153) Bentonite sample contains: Ca, Fe, Na, Mg, Al, Si and S, with Table showing the corresponding atomic% of the elements, in which two different types of sample mounting were explored. Results show no significant difference between the two methods employed for sample mounting i.e. as a thin layer and when powder was packed onto an SEM stub (as shown in Table 1a and b, respectively).

Remarks on results including tables and figures

Attached background material

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf / 5.9 MB (application/pdf): Appendix

Applicant's summary and conclusion

Conclusions

EDX results show that the NM-600 (0153) Bentonite sample contains: Ca, Fe, Na, Mg, Al, Si and S. Compositions are provided in Table above.

Executive summary

Cross-reference to other study

4.32 Dustiness

4.33 Porosity

4.34 Pour density

4.35 Photocatalytic activity

4.36 Radical formation potential

4.37 Catalytic activity

5. ENVIRONMENTAL FATE AND PATHWAYS

6. ECOTOXICOLOGICAL INFORMATION

6.1 Aquatic toxicity

6.1.1 Short-term toxicity to fish

Endpoint study record: Fish, acute toxicity test

Test materials

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

Nanoclay NM-600, vessels no. 0006, 0676, 0691

Test material form**Details on test material****Confidential details on test material*****Details on properties of test surrogate or analogue material*****Method**

OECD 203 (17.07.1992): OECD guideline for testing of chemicals – Fish, acute toxicity test.

Analytical monitoring

The particle size distribution was determined at test start and at day 1, day 2 and day 4 (test end). For sampling a 20 mL volumetric pipette was used. During sampling the pipette was moved through the whole water body. The sample was frozen in glass vessels until the analyses were performed. The particle size distribution was measured using a Malvern Zeta-Sizer. For the zeta- potential the value of the test with daphnids was used. In both tests the same aqueous medium (purified drinking water) was applied. Test concentrations were determined at test start and at day 1, day 2 and day 4 (test end). For sampling a 20 mL volumetric pipette was used. During sampling the pipette was moved through the whole water body. The sample was frozen in glass vessels until analyses were performed.

Details on sampling***Details on analytical methods*****Vehicle*****Details on test solutions*****Test organisms**

Danio rerio is listed in the OECD TG 203 as test organism representing aquatic vertebrates.

Test organisms (species)

Danio rerio (Teleostei, Cyprinidae)

Details on test organisms

Length: 2 cm ± 1 cm

Source: in house culture

Origin of the strain: West Aquarium GmbH 37431 Bad Lauterberg, Germany.

Holding: The fish were reared in water of the same quality as used in the test (purified drinking water) for at least three months until the start of exposure.

Health: The criteria of the test guideline 203 were followed. Only healthy fish without visible diseases and abnormalities were used in the study.

Holding- and dilution-water: Purified drinking water was used as holding- and dilution water. The purification included filtration with activated charcoal, passage through a limestone column, and aeration. To avoid copper contamination, plastic water pipes are used. The following water chemistry data are recorded regularly in the testing facility and are reported: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium (NH₄⁺), phosphate, calcium, magnesium, total hardness, alkalinity, DOC content, content of metals (copper, iron, manganese and

Freshwater Draft Report: Ecotoxicological tests with nanoclay NM-600 zinc).

Study design

Static

Test type

Water media type

Limit test

Total exposure duration

96 h: February 28th - March 04th, 2011

Post exposure observation period

No post-exposure observation period was performed.

Test conditions

Hardness

1.1 mmol/L

Test temperature

22.4 - 22.8 °C (recommended range: 21 - 25 °C)

pH

8.1 – 8.7 (recommended range: pH 6.0 – 8.5)

Dissolved oxygen

About 100% corresponding to about 8.6 mg/L (demanded threshold value: $\geq 60\%$)

Salinity

289 $\mu\text{S}/\text{cm}$

Nominal and measured concentrations

Test concentrations were:

1.0 mg/L, 10.0 mg/L, 100.0 mg/L

The test suspensions were prepared by adding 10 mg, 100 mg and 1000 mg in purified tap water (5 L). Glass vessels were used. The dispersions were stirred (1 min) and treated with ultrasound in an ultrasonic bath (3 min). The suspensions were transferred in full glass aquaria (12 L) and 5 L of purified drinking water added.

Details on test conditions

- Test vessel: glass beakers (12L) filled with 10L test suspension
- Aeration: yes
- Stirring: the aquaria were placed on magnetic stirrers. Stirring was performed with magnetic bars. To avoid damage of the fish the magnetic bars were covered with a wired cage.
- Photoperiod: yes (12/12h)
- Renewal rate of test solution: no
- No. of organisms per vessel: 7
- No. of vessels per concentration (replicates): 1
- No. Of vessels per control (replicates): 1
- Temperature, pH, and oxygen concentration of the water were measured in each vessel directly before adding the fish and afterwards daily.
- Test medium / water parameters
- Purified drinking water was used. The purification included filtration with activated charcoal, passage through a lime-stone column and aeration until oxygen saturation.
- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Vehicle control: no

Reference substance (positive control)

In order to confirm the sensitivity of the test species *Danio rerio* mortality tests over 96 h with the reference substance CuSO₄ are performed in regular intervals. The 96 h LC₅₀-values obtained in this institute are within the range of 0.06 - 0.11 mg/L.

Any other information on materials and methods incl. tables**Results and discussions****Effect concentrations**

No mortality and no effect on the behaviour of the test fish were observed (Tables below). Therefore, no EC_x-values could be determined. The NOEC is ≥ 100 mg/L.

Table: Clinical signs during the test period of 96 h (number of fish with symptoms), 7 fish per concentration were introduced. Concentrations given as nominal concentrations

Incubation	3h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
1 mg/L	0	0	0	0	0
10 mg/L	0	0	0	0	0
100 mg/L	0	0	0	0	0

Table: Cumulative mortality during the test period of 96 h (number of dead fish), 7 fish per concentration were introduced. Concentrations given as nominal concentrations

Incubation	3h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
1 mg/L	0	0	0	0	0
10 mg/L	0	0	0	0	0
100 mg/L	0	0	0	0	0

Details on results

The toxicity of NM-600 on mobility of the species *Danio rerio* was investigated. The fish were placed in water containing the test item in nominal concentrations of 1.0, 10.0, and 100 mg/L. The test was conducted under static conditions for 96 h. Effects on mortality and behaviour were determined after 96 hours. No fish died or showed abnormal behaviour. Therefore, no LC_x-values could be determined. The NOEC is ≥ 100 mg/L.

Results with reference substance (positive control)

In order to confirm the sensitivity of the test species *Danio rerio* mortality tests over 96 h with the reference substance CuSO₄ are performed in regular intervals. The 96 h LC₅₀-values obtained in this institute are within the range of 0.06 - 0.11 mg/L.

Reported statistics and error estimates**Any other information on results incl. tables**

Overall remarks, attachments

Remarks on results including tables and figures

Applicant's summary and conclusion

Validity criteria fulfilled

The test is considered to be valid since:

- Mortality in controls did not exceed 10%.
- Constant conditions were maintained throughout the test.
- The dissolved oxygen concentration was $\geq 60\%$ throughout the test.

Conclusions

Nanoclay NM-600 was tested for mortality of the species *Danio rerio*. A static test with nominal concentrations of 1.0, 10.0, and 100 mg/L was performed. During the incubation the test dispersions were stirred. To avoid damaging of the fish the magnetic bar used for stirring was protected by a mesh. Effects on mortality and behaviour were determined after 96 hours. No fish died or showed abnormal behaviour. Therefore, no LCx-values could be determined. The NOEC is ≥ 100 mg/L.

A high silica concentration was detected in the control samples. This is due to the ubiquitous occurrence of silica (e.g. in dust). In the fish test the suspensions in the test vessels were determined. Due to the high background concentration, the lowest concentration (1 mg/L) could not be determined with sufficient accuracy. During the incubation period of 96 h recovery in the individual test vessels of the two higher test concentrations (10, 100 mg/L) increased indicating an improved distribution of the nanomaterials in the large test vessels due to stirring. Recoveries above 80% were determined after 1 day (10 mg/L) and after 2 days (100 mg/L). Due to the recoveries of 87 and 88% at the end of the test the nominal test concentrations were used for the calculation of NOEC and LOEC values.

Executive summary

The toxicity of the test item nanoclay NM-600 on the mortality of the species *Danio rerio* was investigated. The fish were placed in water containing the test item in nominal concentrations of 1.0, 10.0, and 100 mg/L. The test was conducted for 96 h 5.8 under static conditions. Effects on mortality and behaviour were determined after 96 hours. No fish died or showed abnormal behaviour. Therefore, no LCx-values could be determined. The NOEC is ≥ 100 mg/L.

A high silica concentration was detected in the control samples. This is due to the ubiquitous occurrence of silica (e.g. in dust). In the fish test the suspensions in the test vessels were determined. Due to the high background concentration, the lowest concentration (1 mg/L) could not be determined with sufficient accuracy. For the higher concentrations recovery was higher. During the incubation period of 96 h recovery in the individual test vessels of the two higher test concentrations (10, 100 mg/L) increased indicating an improved distribution of the nanomaterials in the large test vessels due to stirring. Recoveries above 80% were determined after 1 day (10 mg/L) and after 2 days (100 mg/L). Due to the recoveries of 87 and 88% at the end of the test the nominal test concentrations were used for the calculation of NOEC and LOEC values.

Cross-reference to other study

6.1.2 Long-term toxicity to fish

6.1.3 Short-term toxicity to aquatic invertebrates

Endpoint study record: Daphnia sp. Acute Immobilisation Test

Test materials

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

Nanoclay NM-600, vessel no. 0002

Test material form

Details on test material

Confidential details on test material

Method

OECD 202 (13.04.2004): OECD guideline for testing of chemicals – *Daphnia* sp. Acute Immobilisation Test

Details on properties of test surrogate or analogue material

Analytical monitoring

Particle size distribution and zeta potential were determined at test start. Additionally the modification of the particle size distribution was determined at test end (day 2). Zeta-potential and particle size distribution were measured using a Malvern Zeta-Sizer. The Zeta-potential was determined for the control and for the concentration of 11 mg/L. The particle size distribution was measured for every concentration. For chemical analyses, at every sampling time (day 0, 1, 2) and test concentration two samples each with 20 mL were taken from vessels prepared just for these analyses but treated as the vessels used for the ecotoxicological determinations.

Details on sampling

Details on analytical methods

Vehicle

Details on test solutions

Test concentrations were:

1.2 mg/L. 3.7 mg/L. 11.0 mg/L. 33.0 mg/L. 100.0 mg/L

The test suspensions (500 mL) were prepared in purified tap water in 500 mL glass vessels (Schott). Test concentrations of 11 - 100 mg/L were prepared individually. Accuracy of weighting increased the demanded concentration by one position after the decimal point. The test concentration of 3.7 mg/L was prepared by dilution from the test concentration of 100 mg/L (18.5 mL adjusted to 500 mL). The test concentration of 1.2 mg/L was prepared by dilution from the test concentration of 33.0 mg/L (18.2 mL adjusted to 500 mL).

Test organisms

The test organisms were young specimens of *Daphnia magna* (clone V), 4 - 24 hours old at test start.

Test organisms (species)

Details on test organisms

Origin of the daphnids: German Federal Environment Agency (Umweltbundesamt), Institut für Wasser-, Boden- und Lufthygiene. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions:

Adult Daphnia at least 3 weeks old were separated from the stock population by sieving. Batches of 30 to 50 animals were held at room temperature in approx. 1.8 L dilution water for one week. During this week the daphnids were fed daily with an algal suspension (*Desmodesmus subspicatus*) and LiquizellR (HOBBY). Algae growing in the log-phase were centrifuged and the pellet was re-suspended in a few mL of medium. 30 mL of this suspension was given to 1 L Daphnia medium. The water was changed once per week. Newborn Daphnia were separated by sieving, the first generation was discarded.

Holding- and dilution-water:

Purified drinking water was used as holding- and dilution water. The purification included filtration with activated char- coal passage through a limestone column and aeration. To avoid copper contamination plastic water pipes are used for the testing facilities. The following water chemistry data are recorded regularly in the testing facility and reported: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium (NH₄⁺), phosphate, calcium, magnesium, total hardness, alkalinity, DOC content, content of metals (copper, iron, manganese and zinc).

Food:

According to the OECD guideline 202, the daphnids were not fed during the test.

Study design

Static

Test type

Water media type

Freshwater

Limit test

Total exposure duration

48 h: February 8th - February 10th, 2011

Post exposure observation period

No post-exposure observation period was performed.

Test conditions

Hardness

1.1 mmol/L

Test temperature

20.0 - 20.2 °C (permitted range: 20 ± 2 °C)

pH

7.3 – 8.3 (permitted range: pH 6 – 9; variation less than 1.5)

Dissolved oxygen

About 100% corresponding to about 8.6 mg/L (demanded threshold value: 3 mg/L)

Salinity

289 µS/cm

Nominal and measured concentrations***Details on test conditions***

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- Renewal rate of test solution: no
- No. of organisms per vessel: 5
- No. of vessels per concentration (replicates): 4
- No. of vessels per control (replicates): 4
- Additional vessels per test concentration were prepared for chemical analyses.
- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8h
- Light intensity: 564-547 lux

Vehicle control: no

Reference substance (positive control)

In order to confirm the sensitivity of the test species *Daphnia magna* (clone 5), acute immobilisation tests over 24 h with the reference substance (RS) K₂Cr₂O₇ were performed in regular intervals, as proposed by OECD 202.

Any other information on materials and methods incl. tables

Test performance:

Young specimens of *D. magna* of similar age (4 – 24 h) were exposed to five concentrations of the test item under static conditions for a period of 48 h. After the test solution was distributed to the replicate beakers the test organisms were added immediately. The daphnids were exposed without aeration in 50 mL of test solution in numbered glass beakers of 50 mL nominal volume. Apparently dead animals were removed at the daily check.

In each medium pH value (pH-meter, e.g. WTW 535), oxygen concentration (e.g. WTW Digital-Sauerstoff-Messgerät Oxi Digi 550) and temperature (e.g. Digitalthermometer, Roth) were checked in the fresh and in the aged medium.

The numbers of dead animals were visually determined daily and dead specimens (if occurred) were removed. Any abnormalities in appearance and behaviour (if occurred) were recorded. Immobility was determined according to the OECD guideline 202. Specimens which were not able to swim within 15 seconds after gentle agitation of the test vessel were considered to be immobilized (even if they were still moving their antennae).

Data evaluation:

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on the nominal concentrations of the test item.

All statistical calculations were performed using the computer programme ToxRat®.

Results and discussions

Effect concentrations

The mean values for immobilisation per treatment level are listed in tables below. Only in the two highest test concentrations one daphnid died. Therefore, no EC_x-values could be determined. The observed immobilisation is not statistically significant and the NOEC is ≥ 100 mg/L.

Table: Mobile daphnids after 24 h and 48 h. Sum of all replicates [Ind.]. N = 20. Concentrations present nominal concentrations

Incubation	Control	1.2 mg/L	3.7 mg/L	11.0 mg/L	33.0 mg/L	100.0 mg/L
24 h	20	20	20	20	20	20
48 h	20	20	20	20	19	19

Table: Immobilisation after 24 h and 48h. Mean values [%]. Concentrations given in nominal concentrations.

Incubation	Control	1.2 mg/L	3.7 mg/L	11.0 mg/L	33.0 mg/L	100.0 mg/L
24 h	0	0	0	0	0	0
48 h	0	0	0	0	5	5

Physical/Pathological symptoms and changes in behaviour:

Neither significant signs of disease nor stress like discolouration or abnormal behaviour were observed in any replicate. All surviving specimens, up to the highest test item concentration, gave the impression of healthy condition.

Details on results

Results with reference substance (positive control)

Reported statistics and error estimates

Any other information on results incl. tables

Overall remarks, attachments

Remarks on results including tables and figures

Attached background material

NM600-Report-Ecotox_final-IME/pdf 1.3 MB (application/pdf): ENV/JM/MONO(2015)10/ANN1

Applicant's summary and conclusion

Validity criteria fulfilled

The test is considered to be valid, since:

- Mortality in controls did not exceed 10%.
- The dissolved oxygen concentration at the end of the aging period (48 h) was ≥ 3 mg/L in control and test vessels.

Conclusions

Nanoclay NM-600 was tested in the acute test with *Daphnia magna*. Immobilisation was determined. Up to the highest test concentration of 100 mg/L no effect due to NM-600 was detected. The NOEC for immobilisation is ≥ 100 mg/L.

A high silica concentration was detected in the controls. The results show that at day 0 a sufficient recovery was obtained. During the incubation period sedimentation occurred.

Executive summary

The toxicity of NM-600 on immobilisation of the species *Daphnia magna* was investigated. For this, the daphnids were placed in water containing the test item in nominal concentrations of 1.2, 3.7, 11.0, 33.0 and 100 mg/L. The test was conducted under static conditions for 48 h. Effects on immobilisation were determined after 24 and 48 hours. Only in the two highest test concentrations one daphnid died. Therefore, no ECx-values could be determined. The observed immobilisation is not statistically significant and the NOEC is ≥ 100 mg/L.

Cross-reference to other study

6.1.4 Long-term toxicity to aquatic invertebrates

6.1.5 Toxicity to aquatic algae and cyanobacteria

Endpoint study record: Freshwater Alga and Cyanobacteria, Growth Inhibition Test

Test materials

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

Nanoclay NM-600; vessel no. 0001

Test material form

Details on test material

Confidential details on test material

Method

OECD Guidelines for the Testing of Chemicals Test No. 201. OECD guideline for testing of chemicals – Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

Details on properties of test surrogate or analogue material

Analytical monitoring

Particle size distribution was determined at test start. Additionally, the modification of the particle size distribution was followed in separate vessels without algae during the test (day 1, 2, 3).

Zeta-potential and particle size distribution were measured using a Malvern Zeta-Sizer. The Zeta-potential was determined for the control and for the concentration of 11 mg/L. The particle size distribution was measured for every concentration.

At day 0, Si concentrations in the test suspensions were determined.

Details on sampling

Details on analytical methods

Vehicle

Details on test solutions

Test concentrations were:

1.2 mg/L, 3.7 mg/L, 11.0 mg/L, 33.0 mg/L, 100.0 mg/L

The test suspensions (500 mL) were prepared with OECD medium (composition according to OECD TG 01 in 500 mL glass vessels (Schott)). Test concentrations of 11 - 100 mg/L were prepared individually. Accuracy of weighting increased the demanded concentration by one position after the decimal point. The test concentration of 3.7 mg/L was prepared by dilution from the test concentration of 11 mg/L (168.182 mL adjusted to 500 mL), test concentration of 1.2 mg/L was prepared by dilution from the test concentration of 3.7 mg/L (162.162 mL adjusted to 500 mL)

Test organisms

The unicellular green alga *Pseudokirchneriella subcapitata* was chosen by experts as test organism representing freshwater primary producers.

Test organisms (species)

Pseudokirchneriella subcapitata (formerly *Selenastrum capricornutum*), Chlorophyceae, Chlorophyta.

Details on test organisms

Origin: SAG, Culture Collection of Algae at Plant Physiological Institute of the University at Göttingen, Albrecht von Haller Institute, Untere Klarspüle 2, 37073 Göttingen, Germany, Catalogue No 61.81 SAG.

Study design

Growth, static

Test type

Water media type

Algae test medium according to OECD TG 201

Limit test

Total exposure duration

72 h: January 31st - February 3rd, 2011

Post exposure observation period

No post-exposure observation period was performed.

Test conditions

Details on test conditions:

Light intensity: 3723-6945lux

Vehicle control performed: no

Hardness

Test temperature

21.8-22.6°C

pH

pH at test start: 7.38-7.51

pH at test end: 7.66 - 8.13

Dissolved oxygen

Salinity

Nominal and measured concentrations

Details on test conditions

Reference substance (positive control)

The sensitivity of the test organism is routinely checked using 3,5-dichlorophenol as primary standard following internal SOPs. The ErC50 value of 5.18 mg/L is comparable with the result of an international ring test, namely 3.38 mg/L.

Any other information on materials and methods incl. Tables

Cultivation and pre-culture:

The stock cultures were maintained fulfilling the criteria of the OECD guidelines. Three days prior to testing a pre-culture was established in OECD growth medium to obtain exponentially growing algae for the test.

Growth medium:

A sterilized synthetic growth medium (OECD medium) according to OECD 201 was used for culture and preparation of the test medium.

Table: Algal medium according to OECD 201

	[mg/L]		[mg/L]
NaHCO ₃	50	H ₃ BO ₃	0.185
NH ₄ Cl	15	MnCl ₂ x 4 H ₂ O	0.415
KH ₂ PO ₄	1.6	ZnCl ₂	0.003
MgSO ₄ x 7 H ₂ O	15	CoCl ₂ x 6 H ₂ O	0.0015
MgCl ₂ x 6 H ₂ O	12	CuCl ₂ x 2 H ₂ O	0.00001
CaCl ₂ x 2 H ₂ O	18	Na ₂ MoO ₄ x 2 H ₂ O	0.007
FeCl ₃ x 6 H ₂ O	0.064	Na ₂ EDTA x 2 H ₂ O	0.1
		pH, at test start	approx. 7.5-8.0

Test vessels:

Test vessels were 250 mL conical glass flasks covered with silicone-sponge caps. The vessels and caps were sterilized prior to use (autoclaving).

Temperature:

During the exposure period, the incubation temperature was measured daily with a calibrated thermometer in an additionally prepared control vessel which was also incubated.

Light intensity:

The light intensity was measured daily using an illuminance meter LI-189 (LI-COR, Lincoln, USA with radiation sensor) with a cosine (2°) receptor in lux on the level of the surface of the test media.

Determination of growth:

Fluorescence measurements were performed instead of particle counts, since particle measurements cannot always distinguish between algae and nanomaterials / agglomerates. Algal biomass was determined after 0, 24, 48 and 72 h by recording the fluorescence intensity using a BioTek Synergy MX microtiter plate reader. 200 µL aliquots were transferred to microtiter plates prior to measurement.

Test performance:

For preparing the test cultures for the growth test, every flask was filled with 100 mL of the respective test medium. 261 µL of the pre-culture (cell density 3.834×10^6 cells/mL) was added to the test vessels to achieve the initial cell concentration of 10,000 cells/mL. Three replicates were prepared for each concentration and six replicates for the controls.

At test start, the initial cell concentration was calculated based on the cell number of the pre-culture. Cell concentrations were measured using an electronic particle counter (CASY 1 Model TT, Schärfe System, Reutlingen, Germany). During the test, the cell concentrations were determined after 24, 48 and 72 h in samples taken directly from the test vessels.

The culture vessels were incubated at 22 ± 1 °C with a light intensity adjusted to approximately 4440-8880 Lux (60-120 µE m⁻² s⁻¹) prior to the test and during the test. The cultures were oscillated by continuously stirring on a laboratory shaker with 100 rpm (Incubation Shaker Multitron ®, INFORS, Switzerland).

The pH values were measured in an additionally prepared replicate at test start and directly in the test vessels at the end of the test. During the exposure the incubation temperature was measured once a day in an additionally prepared control vessel, which was continuously incubated.

Data evaluation:

- The evaluation of the concentration-effect-relationships and the calculation of the effect concentrations were based on the nominal concentrations of the test item.
- The fluorescence values were calculated in cell counts using a calibration curve.
- The mean value of the cell counts for each concentration plot was used for plotting growth curves.
- Calculation of the percent inhibition of growth rate [r] and yield [y] was performed according to the guideline and listed in a table.
- EC10 and EC50 values were determined together with 95% confidence intervals using Probit-analysis (Finney, 1984) assuming a log-normal distribution of the values by using ToxRat (ToxRat® Professional 2.09).
- The NOEC values for growth rate and yield were determined using the computer programme ToxRat (ToxRat® Professional 2.09). For growth rate and yield normal distribution was checked with the Shapiro-Wilk's Test (normal distribution shown), variance homogeneity was checked with Levene's Test (variance heterogeneity shown), NOEC was determined with Welch test for inhomogenous variances with Bonferroni-Holm adjustment.

Results and discussions

Effect concentrations

The percent inhibition of growth rate and yield depending on nominal test item concentrations are listed in (Table) and shown in Figure. Details on the test results are shown in chapter 3.10.2. A concentration dependent inhibition could be observed. The NOEC values for effects on yield were 3.70 mg test item/L. Per expert judgment, the NOEC for the growth rate was determined to be 33.0 mg/L, as effects below 10% are considered not ecotoxicologically relevant. The effect values for the 72 hour exposure period are compiled in Table. The fluorescence of the algae is not influenced by nanoclay. In the presence of NM

600 (100 mg/L) algae resulted in a fluorescence comparable to algae in mineral medium (Table). Therefore, the observed inhibition is due to nanoclay and represents no false positive result for example by quenching of the signal.

Table: Percent inhibition of growth rate and yield by the test item compared to controls after 72 h

Test item, nominal [mg test item/L]	% Inhibition of growth rate	% Inhibition of yield
1.2	-1.3	-7.0
3.7	-4.5	-5.5
11.0	5.1	12.5
33.0	2.9	9.3
100.0	16.9	33.3

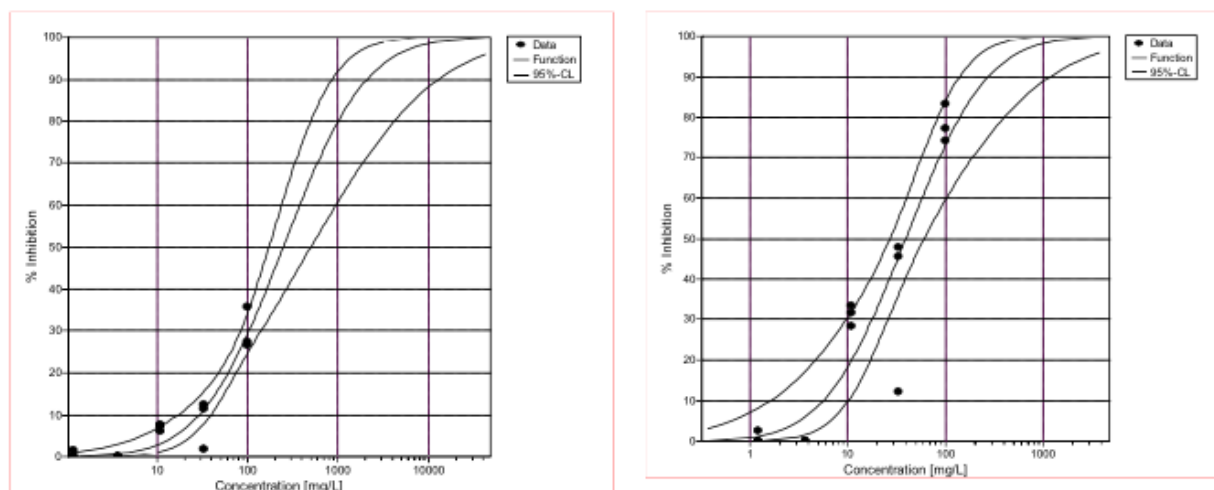


Figure: Concentration-effect curve showing the influence of the mean measured concentrations of NM-600 on % inhibition of growth rate (left) and yield (right) after 72 h.

Table.: Effective concentrations of the test item based on nominal concentrations after exposure to *Pseudokirchneriella subspicata* for 72 hours

		Effective concentrations [mg/L] after 72 h (95% confidence interval [mg/L])		
		EC50	EC10	NOEC
Yield	Nanoclay NM-600	39.23 (27.04-60.64)	5.62 (1.62-10.15)	3.7
Growth rate	Nanoclay NM-600	248.48 ¹ (172.64-502.62)	29.57 (17.26-39.58)	33.3

¹ exceeding the highest test concentration (100 mg/L)

Table: Fluorescence of algae, mineral medium, NM 600

Sample	Fluorescence
Mineral medium (OECD 201)	158 ± 2.7
NM 600 100 mg/L in mineral medium	157 ± 2.4
Algae (about 12 * 10 ⁶ cells/mL; exposure condition at test end) in mineral medium	3621 ± 24.4
Algae in mineral medium + NM 600 (100 mg/L)	3640 ± 24.0

Details on results**Results with reference substance (positive control)****Reported statistics and error estimates****Any other information on results incl. tables****Overall remarks, attachments****Remarks on results including tables and figures****Attached background material**

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Applicant's summary and conclusion**Validity criteria fulfilled**

The alga growth inhibition test fulfills the validity criteria of OECD 201 (2006):

- The fluorescence in the control cultures increased by a factor of 137 within the test period of 72 h (validity criterion for biomass: > 16).
- Evaluation of the sectional growth rates of the controls: The mean of the replicate coefficients of variations in the section-by-section growth rate of controls was 17.7% (validity criterion < 35%).
- The coefficient of variation of average specific growth rate in replicate control cultures during the whole test period was 1.2% (validity criterion < 7%).

Conclusions

Nanoclay NM-600 was tested in the growth test with algae according to OECD test guideline 201. A concentration dependent inhibition of growth rate and yield could be observed.

Executive summary

The toxicity of the test item NM-600 on the growth of the unicellular fresh water green alga *Pseudokirchneriella subcapitata* exposed for a test period of 72 hours under static conditions according to OECD 201 was determined. The test item was suspended in sterilised growth medium. Three replicates for each concentration and six replicates for controls (test medium only) were exposed to nominal 1.2, 3.7, 11.0, 33.0 and 100 mg test item/L. A concentration dependent inhibition of growth rate and yield could be observed. The EC and NOEC values for growth rate and yield are summarised in the following table:

Table: Endpoint data in *Pseudokirchneriella subcapitata* exposed to nanoclay NM-600 for 72 hours based on mean nominal concentrations.

	Effective concentrations [mg/L] after 72 h (95% confidence interval [mg/L])			
		EC ₅₀	EC ₁₀	NOEC
Yield	Nanoclay NM-600	39,23 (27.04-60.64)	5.62 (1.62-10.15)	3.7
Growth rate	Nanoclay NM-600	248.48 ¹ (172.64-502.62)	29.57 (17.26-39.58)	33.3

¹ exceeding the highest test concentration (100 mg/L)

Cross-reference to other study

6.1.6 Toxicity to aquatic plants other than algae

6.1.7 Toxicity to microorganisms

6.1.8 Toxicity to other aquatic organisms

6.2 Sediment toxicity

Endpoint study record: Sediment-Water Chironomid Toxicity Using Spiked Water

Test materials

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

NM-600 (Vessel No 0017 and 0007)

Test material form

Details on test material

Confidential details on test material

Details on properties of test surrogate or analogue material

Method

OECD Guidelines for the Testing of Chemicals Test No. 219. Sediment-Water Chironomid Toxicity Using Spiked Water (2004)

Analytical monitoring

For the control and for each concentration one additional vessel only used for analytical measurements was applied. These additional vessels were treated as the control vessels and the test vessels used for the

assessment of the nanomaterials. At several times aqueous samples (50 mL) were taken at four different depths (about 2.0 cm; 4.0 cm; 5.5 cm; 6.5 cm). The samples were combined. About 20 mL were used for the analyses. The remaining amount was carefully returned into the test vessels without disturbing the sediment.

Zeta-potential and particle size distribution were measured using a Malvern Zeta- Sizer.

Temperature and pH were measured in the overlaying water at test start / end and during the study in all vessels once a week. Dissolved oxygen was measured at test start and twice a week in one representative vessel per treatment; at test end it was measured in all test vessels. Hardness and ammonia were measured in the controls and in one test vessel at the highest concentration at the start and the end of the study.

Test item concentrations in the overlaying water were determined 3 h after adding the test substance, at day 1, 2, 7, 14 and 28. For details of chemical analyses see chapter 7.

Details on sampling

Details on analytical methods

Vehicle

Details on sediment and water

Artificial sediment components:

Sphagnum peat, air-dried, finely ground	5%
Kaolinite, air-dried	20%
Industrial quartz sand, air-dried	75%

The test substrate was wetted with deionised water to reach a water content of about 25% to 30%. According to the Guideline a water content of 30% to 50% is recommended. Our experience shows that a lower water content results in a more homogenous distribution of the sediment in the individual vessels. Pulverized calcium carbonate of chemically pure quality (CaCO₃) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5 . The organic carbon content of the final mixture was 2.2%, which was in the demanded range of $2\% \pm 0.5\%$.

Water: Purified tap water was used as test water.

Details on application

The nominal concentrations in the test containers with the test item were 15, 23, 39, 63, and 100 mg test item/L. Four replicates per concentration were conducted. For each vessel a 500 mL stock dispersion of the nanomaterials in tap water was prepared. For the double concentrated dispersion of the final test concentration the respective amount of nanomaterial was weighted in brown glass vessels using a suitable balance. 500 mL of tap water was added, and the mixture was stirred (magnetic stirrer, 300 rpm) and ultrasonic treatment in a water bath (3 min, 500 W). The stock dispersion was added thoroughly to the water column in the test vessels 24 h after addition of the test specimens. Due to the large amount of stock dispersion, mixing occurred during addition. There was no further mixing to avoid disturbance of the sediment.

Test organisms

The test organisms were the first instar larvae from the dipteran *Chironomus riparius*.

Test organisms (species)

Chironomus riparius.

Details on test organisms

Origin of the midges:

Bayer Crop Science AG, 40789 Monheim, Germany. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions:

On a layer of diatomaceous earth purified tap water was used. The dipterans were fed daily with powder of TetraMin® Hauptfutter (Tetra Werke, Melle, Germany).

Pretreatment:

Four to five days before adding the test organisms to the test vessels, egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20 °C. First instar larvae (1 days post hatching) were used in the test. As the larvae were added one day before spiking the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase).

Study design

Reproduction, static

Test type

Laboratory study

Test duration type

Long-term (28 days)

Water media type

Freshwater

Type of sediment

Artificial sediment

Total exposure duration

The exposure period was 28 days. March 07th - April 4th 2011

Post exposure observation period

No post-exposure observation period was performed.

Test conditions**Hardness**

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

Test end: 120 – 130 mg/L as CaCO₃ equivalents in the controls and 120 mg/L as CaCO₃ equivalents in one representative replicate of the highest test concentration.

Test temperature

20.3 °C (permitted range: 20 ± 2 °C)

pH

8.3 – 8.5 (permitted range: pH 6 – 9)

Dissolved oxygen

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

Ammonia

Test start: 1.2 - 1.4 (control); 1.2 (highest test concentration)

Test end: 4.0 - 6.0 (control); 0.4 (highest test concentration)

Nominal and measured concentrations

The nominal concentrations in the test containers with TiO₂ were 1.2, 3.7, 11, 33, and 100 mg test item/L.

Details on test conditions

The light intensity was measured using an illuminance meter (MINOLTA) with photo- metric sensor in Lux. With 593 – 679 lx the permitted range of about 500 – 1000 lx was kept.

Reference substance (positive control)

According to the guideline a test with a reference substance is not compulsory necessary. 2-Chloracetamid was tested in a sediment-water-chironomid toxicity test using spiked sediment (OECD 218).

Any other information on materials and methods incl. Tables

Control treatment:

The control consists of sediment, tap water and chironomids. Four replicates per control were conducted.

Statistical method:

Data evaluation:

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation. Minor differences in results obtained from calculations with such rounded values in comparison to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

Statistical calculations:

Calculations were performed with the computer software ToxRat Professional version 2.10.4.1 by ToxRat® Solutions GmbH.

Food:

Powder of TetraMin® Hauptfutter was used for feeding the larva. According to the guideline, the food ratios for the first 10 days were 0.25 – 0.5 mg TetraMin® / larvae/day, from day 10 on the food ratio was increased up to 0.5 – 1.0 mg TetraMin® /larvae/day.

Test container:

Round glass beakers (3L) were used as test vessels. The vessels were filled up to 2 cm height with wet artificial sediment (corresponding to 370 g dry mass). The overlaying water was 8 cm high (ratio sediment: water about 1:4). The containers were covered with glass plates. After 10 days, emergence traps were placed on the test vessels, the glass plates remained on the emergence traps to avoid evaporation. Aeration of overlaying water was provided through a glass pipette fixed 2-3 cm above the sediment layer (at least 1 bubble /second).

Test procedure:

Sediment was filled in the test vessels. 400 mL of tap water was added, and the sediment-water system was gently aerated for several days prior to addition of the test organisms. Batches of twenty larvae were placed into each vessel.

After an incubation of 24 h, 500 mL of the freshly prepared stock dispersion of the nanomaterials was added. Further 100 mL of tap water were used to rinse the vessels containing the stock dispersions. To avoid separation of sediment ingredients during the addition of test water and stock dispersion, the surface of the water column was covered with a stainless steel disc while water was poured onto it. The disc was removed immediately afterwards. Due to the large amount of stock dispersion, mixing occurred during addition. There was no further mixing to avoid disturbance of the sediment.

The test was carried out at $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and at 16 hours photoperiod (500 –1000 lux). The exposure duration was 28 days. The development time and total number of fully emerged male and female midges were determined. Test vessels were observed daily for visual assessment of abnormal behaviour. Emergence was counted daily. After identification the midges were removed from the test vessel. At test end, the test vessels were observed for visible pupae that had failed to emerge.

Results and discussions

Effect concentrations

No significant effect on emergence and development rate were observed. Statistical differences were observed for the development time, and NOEC and LOEC values could be determined. However, the differences showed no concentration-effect relationship. The highest concentration showed lower effects than the concentration of 33 mg/L. This might be due to higher agglomeration and lower bioavailability in higher test concentrations. See table below.

Table. NOEC, LOEC and EC10 values for the test with chironomids

	NOEC	LOEC	EC10
Emergence rate of males and females	≥ 100	> 100	n.d.
Development rate of males and females	≥ 100	> 100	n.d.
Development rate of males	≥ 100	> 100	n.d.
Development rate of females	≥ 100	> 100	n.d.
Development time of males and females	≥ 100	> 100	n.d.
Development time of males	≥ 100	> 100	n.d.
Development time of females	3.7	11	n.d.

The NOEC was ≥ 100 mg/L and EC10, EC20, and EC50 values of the biological endpoints were > 100 mg/L.

Physical/pathological symptoms and changes in behaviour:

Neither physical nor pathological symptoms were obtained. All specimens gave the impression of healthy condition. There was only one dead emerged animal in the second concentration (23 mg/L) at day 18.

Details on results

Results with reference substance (positive control)

Reported statistics and error estimates

Any other information on results incl. tables

Overall remarks, attachments

Remarks on results including tables and figures

Attached background material

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Applicant's summary and conclusion

Validity criteria fulfilled

The test is considered valid since

- the mean emergence in controls was 92% (70% mentioned in the guideline) at test end.
- *C. riparius* development time of most adults in controls was between 16 – 23 days after their insertion into the test vessels, which is between the demanded range of 12 – 23 days. However, one further animal developed at day 24, 25 and 26.
- at the end of the test the dissolved oxygen concentration was at least 60% of the air saturation level at the temperature used, and the pH of overlaying water was in a range of 6 – 9 in all test vessels.
- the water temperature differed not more than ± 1 °C between vessels and was maintained within the temperature range of 20 ± 2 °C.

Conclusions

Nanoclay (1.2, 3.7, 11, 33, and 100 mg test item/L) was tested in the chironomid test with spiked water (OECD 219). Nanoclay up to a concentration of 100 mg/L was considered to result in no negative impact on the emergence of larvae in a spiked water sediment test with chironomids. The NOEC is ≥ 100 mg/L.

Executive summary

Nanoclay was tested in the chironomid test with spiked water (OECD 219). The nominal concentrations in the test containers were 1.2, 3.7, 11, 33, and 100 mg test item/L. There was sedimentation of nanoclay resulting in low nanoclay concentrations in the overlaying water. Nanoclay up to a concentration of 100 mg/L was considered to result in no negative impact on the emergence of larvae in a spiked water sediment test with chironomids. The NOEC is ≥ 100 mg/L.

Cross-reference to other study

6.3 Terrestrial toxicity

6.4 Biological effects monitoring

6.5 Biotransformation and kinetics

6.6 Additional ecotoxicological information

7. TOXICOLOGICAL INFORMATION

8. ANALYTICAL METHODS

9. RESIDUES IN FOOD AND FEEDINGSTUFFS

10. EFFECTIVENESS AGAINST TARGET ORGANISMS

11. GUIDANCE ON SAFE USE

12. LITERATURE SEARCH

13. ASSESSMENT REPORTS

14. INFORMATION REQUIREMENTS

APPENDIX

NM600_RT2Report_NANOCLAY_19NOV2010anon.pdf

2. METHODS.

2.1 Materials.

Materials used in this study were as follows:

- a) “as received powders” (~500 mg aliquots) as supplied by the Joint Research Centre (JRC), in which a spinning riffler was used for sample division. These vials (each with individual sample identity codes) were opened and immediately used for analysis, with the remainder being discarded, as recommended by JRC.
- b) “as received Tetra Pak Bentonite slurries” i.e. labelled as sample G (3.3 wt% of Bentonite in DI water) and sample H (0.8 wt% of Bentonite in poly-vinyl alcohol (PVOH) based liquid media).
- c) “as received Tetra Pak films” i.e. cardboard Bentonite labelled as ‘A1, A2 and A3’ (three samples consisting of PVOH, Bentonite and cardboard), OPET Bentonite labelled as ‘C1’ (one sample consisting of PVOH, Bentonite and OPET), peeled off from OPET film labelled as ‘E1, E2 and E3’ (three samples consisting of PVOH and Bentonite); respective films on cardboard and OPET without Bentonite were labelled as B1 and D1 respectively.

2.2 Dispersion and Characterisation Techniques.

The procedure for in-house dispersion of Bentonite in de-ionised water is identical to the dispersion protocol described elsewhere [1]. The various instruments and corresponding protocols employed for nanomaterials (NM) characterisation are outlined in Appendix 1. Apart from the methods associated with XRD and TEM imaging, methods employed here are those based on the protocols used in the PROSPECt project [2] for the characterisation of engineered nanomaterials, (in particular cerium oxide and zinc oxide).

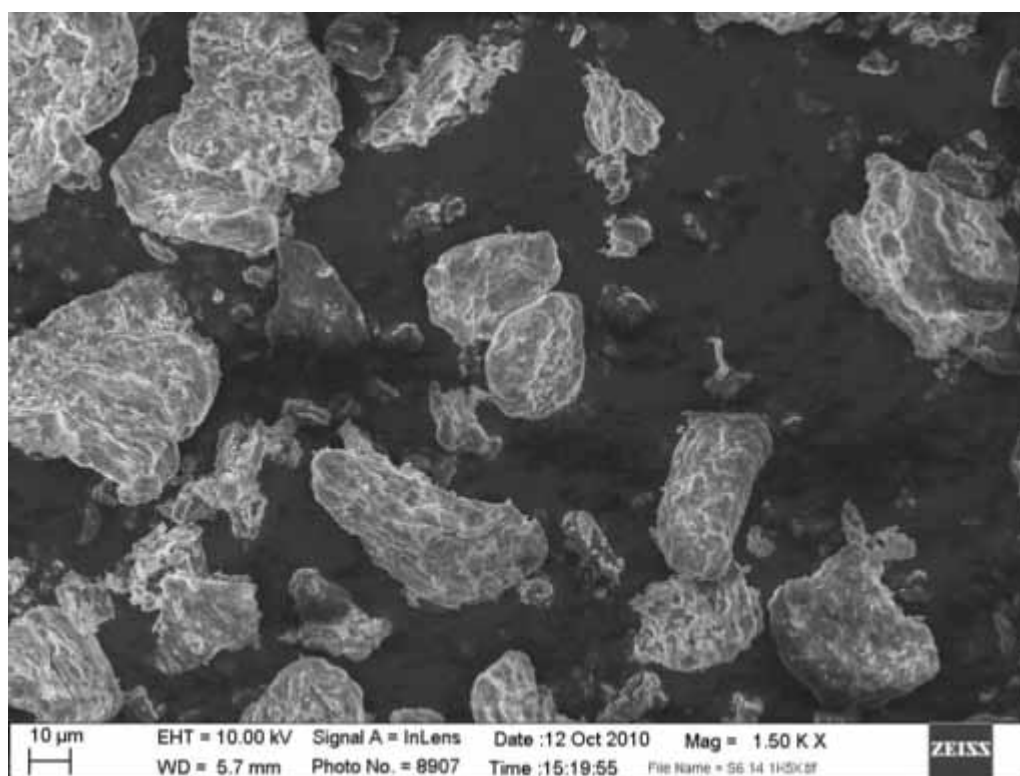
3. RESULTS AND DISCUSSION.

3.1 Characterisation of the “as-received powders”.

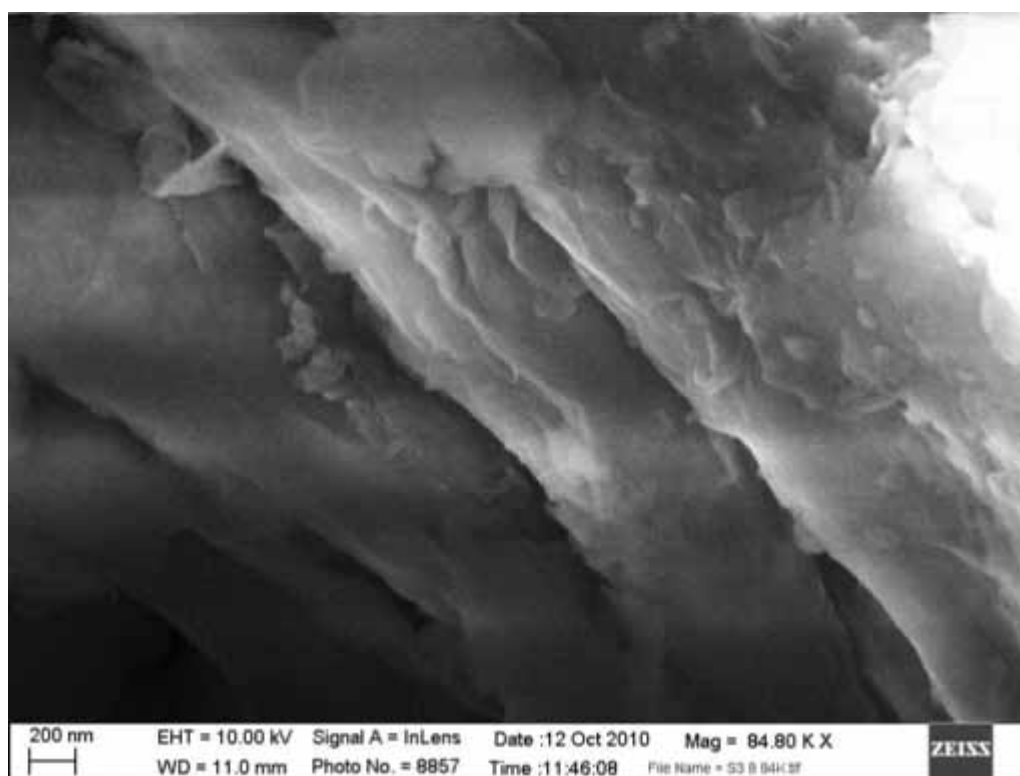
The BET result shows that the specific surface area of the Bentonite clay (NM600, 0073) was 51.9 ± 1.6 m²/g; errors (2 SD) reported here are the uncertainty of the result when acquiring a “one sample” dataset as reported by the BET gas adsorption technique.

Figure 1 shows a typical SEM image of the “as received” Bentonite clay powders at low and high magnification. Images show that:

- c) Particles are highly aggregated/agglomerated, with tens of micron size particles evident.
- d) Each particle is made up of sheet-like units, held together.



a)



b)

Figure 1: SEM images of NM-600 (0153) Bentonite at: a) Low and b) High magnifications.

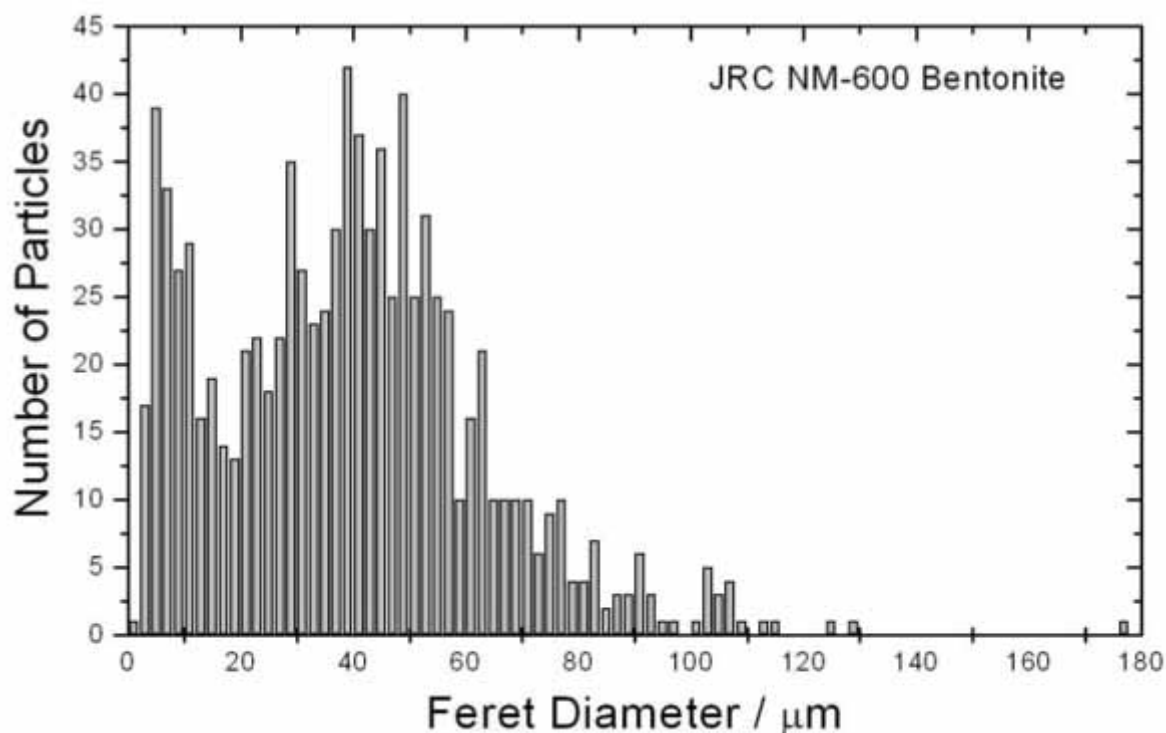
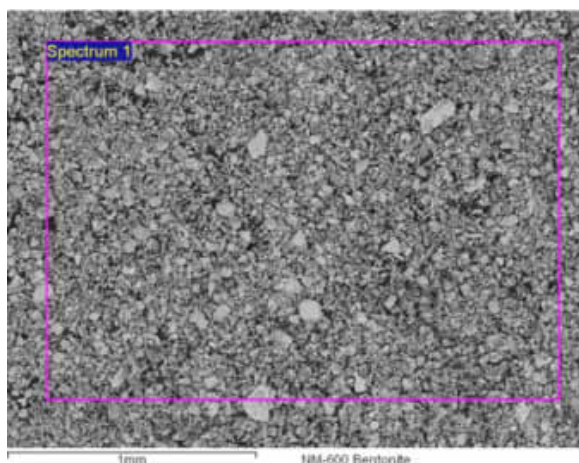


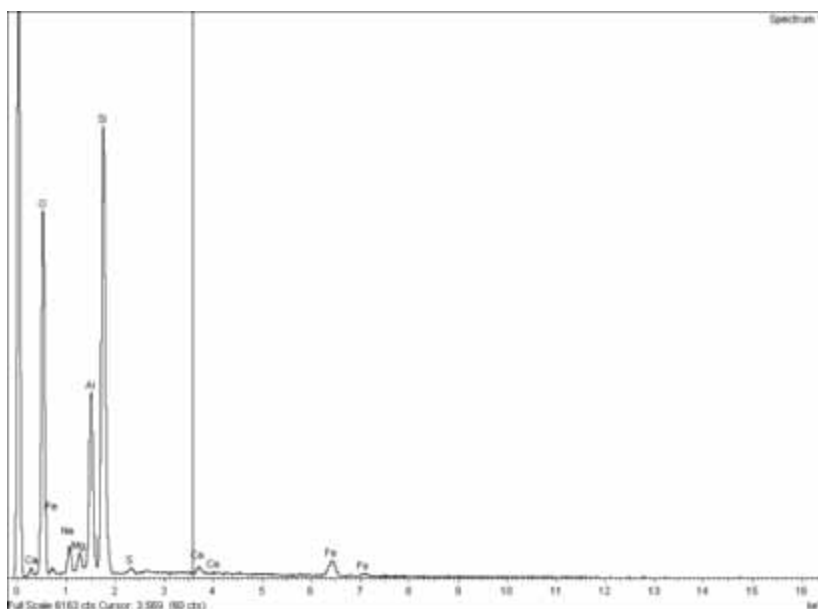
Figure 2: Particle size distribution of Bentonite clay NM-600 (0153), as obtained from the SEM images and analysing ~ 900 clay particles.

Figure 2 shows the particle size distribution of Bentonite clay NM-600 (0153), in which the clay particles are present in the form of clustered sheets of particles. The corresponding mean Feret's diameter of the particles is $39.5\mu\text{m} \pm 23.8 \mu\text{m}$; the SD value reported here gives an indication of the polydispersity. It is evident that the clay sample is highly polydispersed i.e. broad size distribution. Here, we report Feret's diameter, as this parameter is widely used for the analysis of irregular shaped particles. The Feret diameter can be defined as the "maximum calliper length" i.e. the longest distance between any two points along the selection boundary [3].

Figure 3 shows the EDX analysis of NM-600 (0153) Bentonite performed on the selected marked areas.



a)



b)

Figure 3: EDX analysis of NM-600 (0153) Bentonite showing typical: a) selected area for EDX analysis, as marked by the pink box. b) EDX spectrum.

EDX results show that the NM-600 (0153) Bentonite sample contains: Ca, Fe, Na, Mg, Al, Si and S, with Table 1 showing the corresponding atomic% of the elements, in which two different types of sample mounting were explored. Results show no significant difference between the two methods employed for sample mounting i.e. as a thin layer and when powder was packed onto an SEM stub (as shown in Table 1a and b, respectively).

Table 1: Elemental composition (atomic%) according to EDX analysis of NM-600 (0153) Bentonite by stoichiometry (oxygen) of sample that was: a) applied as a thin layer on to an SEM stub b) packed into a specially adapted SEM stub. Table shows the average value (and corresponding 1 SD) from 12 replicate spectra.

a)

	Composition / atomic%								
	Na	Mg	Al	Si	S	Ca	Fe	O	Sum
Average	2.85	1.29	8.61	22.25	0.27	0.29	1.61	62.84	100.00
SD	0.38	0.10	0.22	0.37	0.21	0.10	0.31	0.15	

b)

	Composition / atomic%								
	Na	Mg	Al	Si	S	Ca	Fe	O	Sum
Average	2.79	1.30	8.51	22.16	0.39	0.37	1.57	62.90	100.00
SD	0.51	0.11	0.40	0.70	0.39	0.20	0.42	0.21	

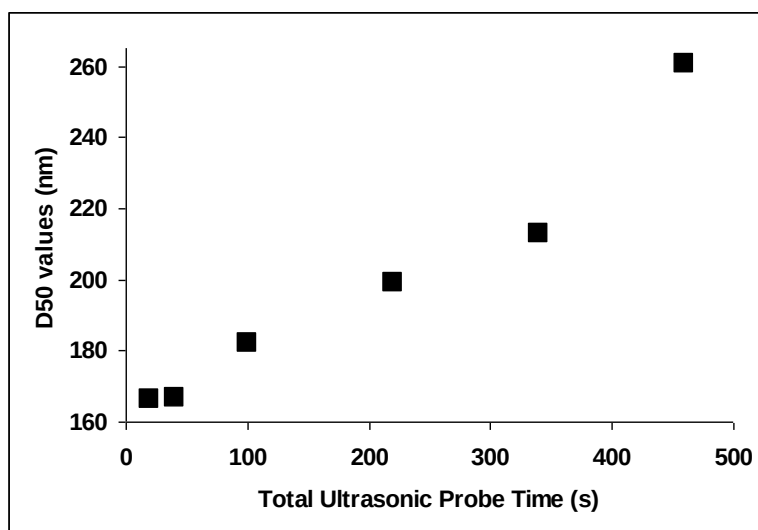
3.2 Characterisation of the as received powders dispersed in DI water, using in-house dispersion protocol.

Table 4 shows the effect of ultrasonic probe exposure time on particle size (where mean particle size, D_{10} , D_{50} and D_{90} values are displayed) of NM-600 (0052) BENTONITE in DI water (3.3 wt% i.e. the same particle concentration as sample G1, as detailed above), as measured by CPS Disc Centrifuge; the dispersion here was carried out in-house in accordance with the procedures and guidelines as detailed in the NIA website.

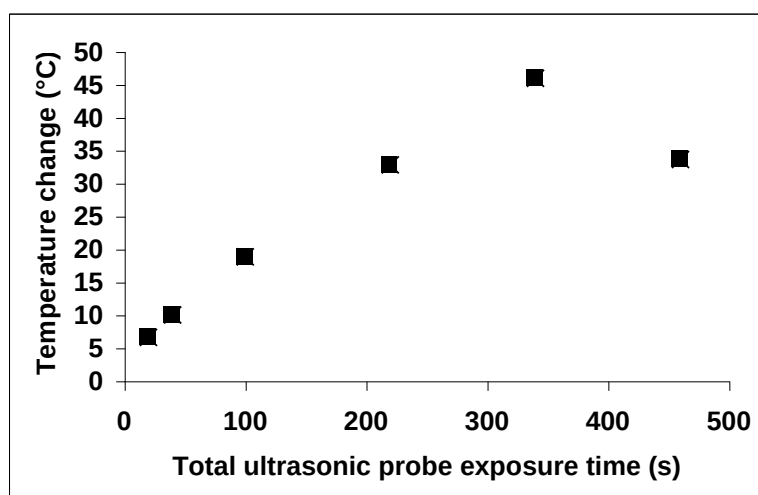
Table 2: CPS disc centrifugal sedimentation results showing: the equivalent mean spherical particle diameter of dispersed NM-600 BENTONITE 0052 and the corresponding D_{10} , D_{50} , D_{90} values (oversize percentiles) from the averaged CPS measurements.

Total Ultrasonic Probe Exposure Time/ (s)	Mean particle size (nm)	D_{10} (nm)	D_{50} (nm)	D_{90} (nm)
20	288	631	166	87
40	282	646	167	83
100	383	1130	182	87
220	407	1123	199	90
340	396	1022	213	94
460	506	1320	261	93

Results indicate that as we increase total sonication time, both mean particle size and the corresponding oversize percentiles (D_{10} , D_{50} , D_{90}) increases. In particular, this trend is very clear when D_{50} values are plotted against total ultrasonic probe time (Figure 6a), in which an almost linear relationship exists between the two variables.



a)



b)

Figure 4: Effects on dispersion characteristics of NM-600 (0052) BENTONITE dispersed in DI water, using the recommended dispersion protocol, as a function of total ultrasonic probe exposure time on: a) D_{50} values b) temperature change measured in the dispersion.

Results indicate that the clay aggregate/agglomerate, resulting in the formation of larger particle sizes, increases with sonication treatment time. The increase in sonication treatment time is also accompanied by an increase in the temperature of the dispersion, which is hardly surprising (Figure 6b). The increase in temperature can promote further aggregation/agglomeration amongst the particles. A rather strange effect is observed when total exposure time is 460s, in which the temperature change in the dispersion started to decrease.

Figure 7 shows the particle distribution (of particle spherical equivalent against relative weight percent) of our in-house dispersion (3.3 wt% of Bentonite 0052, dispersed in DI water), as acquired using the CPS disc instrument. The plot also shows the corresponding size distribution of the Tetra Pak sample (G1 i.e. 3.3 wt% in DI water).

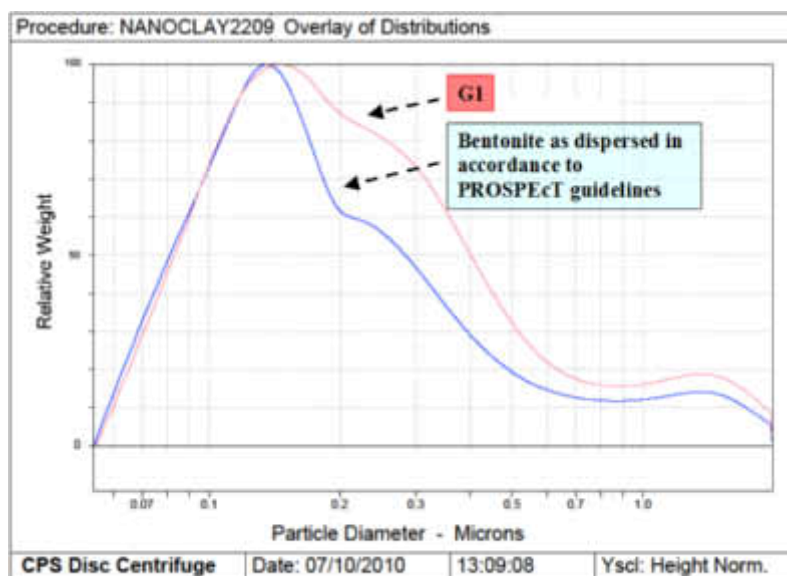


Figure 5: Dispersion characteristics of G1 vs. Bentonite NM-600 BENTONITE 0052 dispersed in DI water, compared with corresponding Tetra Pak G1 sample.

Results show some similarities between the particle distribution profiles but there are certain differences. The shoulder peak apparent (at $\sim 0.2 \mu\text{m}$) is more pronounced with our dispersion rather than the corresponding Tetra Pak G1 dispersion. This indicates a broader particle size distribution in the case of the Tetra Pak G1 sample.

Table 5 displays the value of “half-lives” of the Bentonite dispersion (NM-600 Bentonite in DI water (dispersed with ultrasonic probe exposure time of 20 and 460 s) vs. the Tetra Pak G1). The concept of “half lives” has been put forward by the OECD guidelines as a parameter indicative of dispersion stability through time i.e. here, half life equates to the time it takes for particle concentration to reduce by half, as determined by turbidity measurements. Hence, the larger the half-life value the longer it takes for particles to sediment out and thus the better the dispersion stability.

Table 3: Dispersion stability as measured by their corresponding “half lives” of Bentonite Nanoclay. The half-life values of Bentonite NM-600 (0053) dispersed in DI water (using in-house dispersion protocol) was compared to the “as received” slurries from Tetra Pak (G1 sample).

Sample	Description/ and Batch number	Half-life values (mins)	Half-life values (hours)
Bentonite NM-600 (0053)/ with ultrasonic exposure time of 20 s	Dispersed using in-house dispersion protocol; ultrasonic probe exposure time 20 s	6204	103
Bentonite NM-600 (0053)/ with ultrasonic exposure time of 460 s	Dispersed using in-house dispersion protocol; ultrasonic probe exposure time 460 s	14760	246
Bentonite Tetra Pak G1	As received dispersion	9840	164

Results show that NM-600 Bentonite in DI water, as dispersed using our in-house dispersion protocol with a 20 s exposure time, has a much smaller half life value when compared to Tetra Pak's G1 sample. This suggests that this dispersion is less stable and thus sediments out at a faster rate. However, when total sonication exposure time in our dispersion was increased from 20 s to 460 s, this had a dramatic effect on the corresponding half-life value, in that the dispersion became less prone to sedimentation. In fact, the 460 s exposure resulted in a much larger half life value compared to Tetra Pak's G1 sample; this is indicative of the fact that our in-house protocol with 460 s ultrasonic exposure time had resulted in a more stable dispersion than the corresponding Tetra Pak's G1 sample. Certainly, results indicate that exposure time governs the overall stability of the dispersion. It is likely therefore that increasing the exposure time can result in increasing dissolution process of the clay in the DI water; this can potentially change the viscosity of the dispersion i.e. making it more viscous. The more viscous the solution the less prone the dispersion will be to sedimentation.

4. CONCLUSIONS.

This report describes the results from the characterisation of the Bentonite Nanoclay, using various techniques, of the: 'as received' NM-600 powders, 'as received' Tetra Pak slurries and Tetra Pak films. Several interesting points to note from the study:

- a) Dispersion using our in-house dispersion protocol resulted in different particle size distribution and dispersion stability, when compared to the corresponding Tetra Pak's slurry. In addition, the particle characteristic changes to a large extent depending on total sonication exposure time.

Main techniques and corresponding protocols.

Parameter	Technique	Protocol
Particle Size & size distribution	CPS disc	Particle size distribution by centrifugal sedimentation was acquired using CPS Disc Centrifuge Model DC 20000 instrument (Analytik Ltd, UK). At the start of the method, the centrifuge was brought up to speed by partially filling the disc with a sucrose gradient fluid and dodecane cap fluid. The purpose of the gradient fluid was to stabilise the sedimentation; the purpose of the cap fluid was to maintain the gradient inside the disc. The disc centrifuge was then allowed to equilibrate at 6000 rpm for 1 hour. This gradient is stable over a 6-hour period. Prior to testing, the slurries were diluted in the corresponding blank media, with a ratio of 1:10 of slurry: blank media. This dilution step was necessary to provide the optimum signal-to-noise ratio. 0.1 ml of the sample was injected into the disc; a calibration standard was injected after every three samples. Analysis was run against a calibration standard (NIST traceable standard, PVC 0.377 μm). The Disc Centrifuge Control System software (CPS Instruments Inc.) was used to acquire and process the data.
	High Resolution SEM	SEM images were obtained using a Supra 40 field emission scanning electron microscope from Carl Zeiss (Welwyn Garden City, Hertfordshire, UK), in which the optimal spatial resolution of the microscope was a few nanometres. In-lens detector images were acquired at an accelerating voltage of 15 kV, a working distance of ~ 3 mm, and a tilt angle 0° . For analysis of the “as received” nanoparticle powder, a sample of the powder was sprinkled over a SEM carbon adhesive disc; one side of the carbon disc was placed securely on a metal stub, whilst the other side was exposed to the nanoparticle powder. Excess powder was removed by gently tapping the stub on its side until a light coating of powder on the surface became apparent. Samples were thinly sputtered with gold using an Edwards S150B sputter coater unit (BOC Edwards, UK). Sputtering was conducted under vacuum (~ 7 mbar or ~ 0.7 mPa), while passing dry argon into the coating chamber. Typical plate voltage and current were 1200 V and 15 mA, respectively. The sputtering time was approximately 10s, which resulted in an estimated gold thickness of not more than 2 nanometres being deposited on top of the substrate. An adequate magnification was chosen for image acquisition e.g. for the estimation of primary particle mean diameter and the estimation of aggregates/agglomerates. The shape and limits of the primary particles and agglomerates/aggregates should become apparent. SEM micrographs were analysed by manually tracing contours of primary particles on to a transparency sheet. The transparency sheet was scanned for further image analysis using ImageJ software, which automatically calculated particle diameter dimensions.

Elemental Analysis	EDX	The JRC NM-600 Bentonite (Sample Identification Number = 0153) was mounted in two different ways for SEM examination: 1. Sample sprinkled as a thin layer onto an adhesive carbon disk 2. Sample packed into a specially adapted SEM stub. Energy dispersive X-ray (EDX) microanalyses were carried out in a <i>Camscan MX2500</i> SEM (serial number: 149, gun type: thermionic emission / W-filament) fitted with an <i>Oxford Instruments Ltd. "INCA Energy+"</i> EDX system (Si-Li EDX detector serial number: 14117-3390-339-51). The SEM/EDX conditions were set to the following standard operating conditions: 20kV accelerating voltage, 35 mm working distance, 50 s count time, 23 - 25 % detector dead time and process time: 5 μs. The beam current was adjusted to give approximately 3000 counts per second (cps) on a pure cobalt (Co) specimen (gain calibration). EDX spectra were acquired at various magnifications in the range: x20-x50 and scanning the electron beam in selected rectangular areas within a pre-selected visual field. All EDX spectra were processed using an <i>Oxford Instruments Ltd. 'Inca Energy+'</i> ZAF-matrix correction program called <i>XPP</i>, (where: Z-atomic number, A-absorption and F-fluorescence). In EDX microanalysis, it is not possible to determine quantitatively the concentrations of the light elements ($Z < 11$) such as C, N and O from the measured spectra with reasonable accuracy due to instrumental factors, the type of material being analyzed and inaccurate ZAF corrections. For $Z \geq 11$ (Na), the EDX detector has an approximate minimum detection limit of 0.1wt%. Hence, the concentrations were calculated using known stoichiometric combinations of the detected elements with oxygen e.g. Na₂O, CaO, Fe₂O₃ etc.
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Dispersion stability	Turbidity reading as a function of time	Turbidity was measured using HF Scientific – Micro100 RI turbidity meter (Cole-Palmer, UK); this meter has an infrared light source that meets the international standard ISO 7027 for turbidity measurements. The meter was calibrated on standards, which are based on AMCO-AEPA-1 microspheres; these standards are traceable to standard formazin suspension. Standard values of 1000, 10 and 0.02 NTU were used to calibrate the meter. Prior to use, the meter was allowed to warm up for 30 minutes. Sample cuvettes (HF Scientific (USA)) were used to hold the sample. Note that glass thickness may vary from cuvette to cuvette and within the same cuvette. Hence, individual vials were indexed; indexing of the cuvette entails finding the point of the cuvette that light passes through that gives the lowest reading; once indexed the holder can be marked accordingly. Prior to their use, cuvettes were cleaned, in accordance to manufacturer's instructions. This involved washing the interior and exterior of the cuvette with a detergent (2% Hellmanex in DI water); it was then rinsed several times in distilled water before finally rinsing in DI water. The cuvette was further rinsed with the sample two times before filling (30ml) and analysed. The cuvette was placed into the meter and signal allowed to settle before taking readings. Prior to testing the slurries were diluted in the corresponding blank media; a ratio of 1:10 of slurry: blank media. The measurement signal was compared with corresponding slurries with no dilution step involved. It was observed that this dilution step was required in order to avoid saturation of the turbidity signal. Air bubbles from the samples were removed by placing the sample under vacuum for several hours. Turbidity readings were taken at regular time intervals.
	Zeta-potential	Electrophoretic measurements were obtained using a Zetasizer Nano ZS (Malvern Instruments, UK) equipped with a 633 nm laser. The reference standard (DTS1230, zeta-potential standard from Malvern) was used to qualify the performance of the instrument. Sample preparation involved filling of a disposable capillary cell (DTS1060, Malvern). Prior to their use, these cells were thoroughly cleaned with ethanol and de-ionised water, as recommended by the instrument vendor. For analysis, the individual cell was filled with the appropriate sample and flushed before re-filling; measurement was carried out on the second filling. Malvern Instrument's Dispersion Technology software (Version 4.0) was used for data analysis and zeta-potential values were estimated from the measured electrophoretic mobility data using the Smoluchowski equation. Prior to testing, the slurries were diluted in the corresponding blank media; a ratio of 1:10 of slurry: blank media, in order to optimise the signal collected. This was done for both the DI water and PVOH based media. For the latter, measurement signal was compared to corresponding slurry with dilution factor of 1:2 (of slurry: blank). Dilution of the PVOH based slurries was done using a pipette tip and any bubbles in the dispersion were subsequently degassed (by placing the sample under vacuum). Samples with no air bubbles were extracted and placed into the zeta-potential cell.

Specific surface area	BET	BET surface area measurements were determined using Autosorb-1 (Quantachrome Instruments). The Autosorb-1 was calibrated using a quartz rod of a known volume, which is traceable to NIST. This calibration was then further checked using two BAM certified reference materials: BAM-PM-102 (nominal SSA $5.41\text{m}^2\text{g}^{-1}$) and BAM-PM-104 (nominal SSA $79.8\text{ m}^2\text{g}^{-1}$). These two reference materials allowed the range of SSA of the nanoparticles to be encompassed with known specific surface area materials, thus adding confidence to the measurements. Surface area measurements were acquired using an 11-point BET gas adsorption method, with nitrogen as the adsorbate. Prior to analysis, the powdered sample was transferred to a sample bulb, then sealed and subsequently degassed overnight at 300°C under a high vacuum and subsequently weighed on a analytical balance in order to determine the sample mass after the degassing step.
Layered distances	TEM	For TEM analysis, a small thin (not more than 200 nm) section (also called a foil) was prepared. The need to produce extremely thin sections presented unique challenges. Initial attempts to section the cardboard sample (containing the Bentonite) using an ultramicrotome (at -80°C) failed, when the material completely broke down. The procedure was repeated with the bentonite-PVOH-OPET sample; this was shown to produce sections of low quality, due to the delamination of the film from the substrate. Only the PVOH-bentonite sample (i.e. free standing films of E1, E2 and E3) could be employed for TEM analysis. For such films, the sample preparation involved suspending the films into an epoxy resin (Struers Epofix). Small sections were successfully sectioned, at room temperature, into thin foils (using a Leica UC7 ultramicrotome); the sections produced by this method showed the highest quality in terms of having consistent thickness. The thickness was varied so that each section had a gold-silver coloured appearance, which indicated a thickness of less than 100 nm. The foils produced were floated onto water and collected using a standard TEM copper mesh. All images were taken using a Hitachi 2300A instrument operated at 200 kV. This instrument is based at the University of Surrey, Guildford and the analysis was carried out by NPL personnel.

5. REFERENCES.

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2. NIA, <http://www.nanotechia.org/>.
3. Chang, H.W. and Okuyama, K., *Optical properties of dense and porous spheroids consisting of primary silica nanoparticles*. Journal of Aerosol Science, 2002. 33(12): p. 1701-1720.