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Nanotechnologies — Toxicity assessment and bioassimilation of manufactured nano-objects in suspension using the unicellular organism Tetrahymena sp.

Nanotechnologies — Évaluation de la toxicité et de la bioassimilation des nano-objets manufacturés en suspension à l'aide de l'organisme unicellulaire Tetrahymena sp.

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 229 *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

In recent years, many studies have been carried out to investigate the effect of manufactured nano-objects (MNOs) on aquatic organisms and their ecosystem. Development and more common use of MNOs in consumer products lead to an increased exposure, and hence a higher possibility of impact on human health and the environment, in case the MNO cause adverse effects. Nanoparticles are used for example in various household products, industrial processes, and in products spanning applications from construction to health and fitness, and MNOs can end up in the environment, for example, bound to wastewater sludge, ultimately entering into the aquatic environment.

Various aquatic organisms (such as fish, daphnia, artemia, algae) are currently used to predict the potential harmful effects of chemicals, including MNOs, on the aquatic environment. Unicellular protozoa of the genus *Tetrahymena* sp. are freshwater organisms with widespread distribution in aquatic environments and are at the bottom of the aquatic food chain. *Tetrahymena* sp. (Protozoa, Ciliata, Oligohymenophorea) are non-pathogenic, free-living eukaryotes and ubiquitously distributed in nature and constituting an important connection between the highly productive and nutrient retaining microbial loop and the metazoans of the classical food chain. This unicellular eukaryote which is bigger than many mammalian cells (approximately 30 µm to 50 µm), can be found in temperate freshwater environments and exhibits nuclear dimorphism (two types of cell nuclei). They have a larger, non-germline macronucleus and a small, germline micronucleus. *Tetrahymena* sp. has a fast generation time, shows a high level of complexity and it is a typical eukaryotic cell resembling cells in multicellular organisms including humans. In addition, although it is unicellular, it possesses many core processes conserved across a wide diversity of eukaryotes (including humans) that are not found in other single-celled model systems (e.g. the yeasts *Saccharomyces cerevisiae*).

The protozoan *Tetrahymena* sp. is an established experimental model in biological studies and it has been extensively used for more than six decades as a toxicological model organism to test the toxicity of different substances using several endpoints.[12] During the last several years, considerable effort has been devoted to computational modelling of the toxicity of chemicals to *Tetrahymena pyriformis* for medium and large sized data sets using computational modelling.[27] It means that data from standardized tests is highly needed. In recent years, viability of cells of *Tetrahymena* sp. has been suggested also as a routine test of MNOs toxicity.[1]-[24] There are several advantages to using *Tetrahymena* sp. as a biological model for a toxicological test model system in freshwater aquatic toxicology and in bioassimilation experiments:

— abundant information is available about using *Tetrahymena sp*. in cellular biology, ecology and ecotoxicology and its role in the microbial food web;

— cells of *Tetrahymena* sp. can easily be cultured at high densities;

— *Tetrahymena* sp. possesses features of both single eukaryotic cells and whole organisms;

— *Tetrahymena* sp. plays an important role as grazers of microbes in aquatic environments and balancing bacterio-plankton production;

— *Tetrahymena* sp. has acceptable sensitivity to exposure to different xenobiotics;

— some species of Tetrahymena possess a genetically fully sequenced macronucleus, thus facilitating the study of changes in gene expression patterns under pollution stress (toxicogenomics);

— *Tetrahymena* sp. is an invertebrate, lacks the characteristic of vertebrates but can still be used to replace the use of animals in toxicity testing at initial stages of testing;

— *Tetrahymena* sp. eats anything that fits into their mouth; it has a highly developed system for the internalization of nanoscale and microscale particles which makes them an ideal model system in nanotoxicity and material cellular internalization (bioassimilation) research.

To ensure the sustainable development of nanotechnology, there is a need for hazard identification and risk assessment of MNOs. This document provides a standard protocol intended to generate reliable

toxicity and bioassimilation data by using *Tetrahymena* sp. for evaluation of MNOs in any experimental suspension of MNOs of interest or in samples from freshwater ecosystems.

Tetrahymena is positioned as a primary consumer in the freshwater food chain, so it is considered as a potential vehicle of environmental contaminants. *Tetrahymena* phagocytic activity is a cost-effective, suitable and rapid assessment tool towards cell internalization (uptake and possible assimilation) of pollutants including particles.[4] It can act as a very early and sensitive indicator for the toxic effect of various xenobiotic compounds as well as an indication of internalization / bioassimilation of xenobiotics. The effect of MNOs on *Tetrahymena* can be induced by the ingested (phagocytosed) MNOs, but also by the contact with MNOs (without internalization) or by the metal ions released from metal-containing MNOs in the suspension. The effect of ingested (phagocytosed) material is measured via cell viability (endpoint of effect) measurements. Phagocytic activity is particle internalisation by cells which, in this case, can be measured by the number and appearance of food vacuoles. Detection of MNOs in living cells exposed to a suspension indicates that the suspension contains MNOs that can be internalized by living cells. This can be taken as a characteristic of biological significance of a suspension containing NMOs. “Biological significance” in this case means that material can be internalized (phagocytosed) by cells. In case of exposure to MNOs, the number and appearance of food vacuoles can also be used as a measure that particles of a defined size (which fit into their mouth) are present in a suspension. This can be used as a biological indication of exposure and in parallel the effects of ingested material can be studied. *Tetrahymena* sp. possesses features of both single eukaryotic cells and whole organisms. Several studies have highlighted their potential as models in in vitro toxicological assessment of chemical pollutants using various endpoints. *Tetrahymena* based pilot ring test has been initiated by the German Federal Environmental Agency for ecological risk assessment[11] and further elaborated by OECD for activated sludge.[26] Although the OECD’s working party on manufactured nanomaterials has recently reviewed the relevance of its various test guidelines on traditional experimental models for the testing of MNOs (see Reference [31]), Reference [31] did not review any methods that utilize the *Tetrahymena* sp. phagocytic activity, as mentioned earlier, is a cost-effective physiological endpoint, which can act as a very early and sensitive indicator for the toxic effect of various xenobiotic compounds as well as an indication of internalization or bioassimilation of xenobiotics. In case of MNO exposure, this endpoint can also serve as a measure of exposure to MNOs in any suspension of MNOs where their cellular internalization is of interest.

Nanotechnologies — Toxicity assessment and bioassimilation of manufactured nano-objects in suspension using the unicellular organism Tetrahymena sp.

1 Scope

This document provides a reliable and repeatable method for simultaneous assessment of both exposure and toxicity of manufactured nano-objects (MNOs) using *Tetrahymena* sp. The ingested, internalized material (MNOs) indicates aquatic exposure.

This document is intended to be used by all the centers working with nano(eco)toxicity of MNOs and capable of culturing of *Tetrahymena* sp. The method uses *Tetrahymena* sp. to assess exposure and effects of MNOs. In addition, the test can be used by centers (laboratories) interested in investigating the biological interaction of MNOs with living cells.

This method is applicable to nano-objects such as nanoparticles, nanofibres of certain size (in a µm size range), nanoplates, as well as their aggregates and agglomerates.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 80004(all parts), *Nanotechnologies — Vocabulary — Part 1: Core terms*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 80004 (all parts) and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
agglomerate
collection of weakly or medium strongly bound particles where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding agglomerates together are weak forces, for example van der Waals forces, or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.4]

3.2
aggregate
particle (3.7) comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.5]

3.3
stock suspension
concentrated suspension that will be diluted to some lower concentration for actual use

[SOURCE: ISO/TS 20787:2017, 3.7]

3.4
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-2:2015, 2.1]

3.5
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.4)

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-2:2015, 2.2]

3.6
nanoparticle
nano-object (3.5) with all external dimensions in the *nanoscale* (3.4) where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than three times), terms such as nanofibre or *nanoplate* (3.9) may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

3.7
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general definition of particle applies to *nano-objects* (3.5).

[SOURCE: ISO/TS 80004-2:2015, 3.1]

3.8
nanofibre
nano-object (3.5) with two external dimensions in the *nanoscale* (3.4) and the third dimension significantly larger

Note 1 to entry: The largest external dimension is not necessarily in the nanoscale.

Note 2 to entry: The terms nanofibril and nanofilament can also be used.

Note 3 to entry: See *nanoparticle* (3.5), note 1 to entry.

[SOURCE: ISO/TS 80004-2:2015, 4.5]

3.9
nanoplate
nano-object (3.5) with one external dimension in the *nanoscale* (3.4) and the other two external dimensions significantly larger

Note 1 to entry: The larger external dimensions are not necessarily in the nanoscale.

Note 2 to entry: See *nanoparticle* (3.5), note 1 to entry.

[SOURCE: ISO/TS 80004-2:2015, 4.6]

3.10
sample
one or more sampling items intended to provide information on the population or on the material

3.11
endpoint
recorded observation of a study conducted to determine if a substance has any associated hazards

Note 1 to entry: Endpoints in toxicity studies are measured parameters at different levels of biological complexity (mortality, behaviour, reproductive status, physiological, biochemical changes, etc.)

3.12
median effective concentration
concentration at which there is an effect on 50 % of the organisms in line with the test criterion

[SOURCE: ISO 15088:2007, 3.3, modified — Note 1 to entry has been deleted.]

3.13
50 % impairment growth concentration
concentration of a substance that inhibits 50 % of the growth of the test population (i.e. *Tetrahymena* sp.) within a designated period (i.e. 24h)

3.14
bioassimilation
absorption or adsorption and digestion of food or nutrients by an organism, which is the state or condition of being absorbed or adsorbed into the organism

4 Abbreviated terms

ATP	Adenosine triphosphate
CCD	Charge-coupled device
DDW	Double distilled water
DLS	Dynamic light scattering

EC_{50}	Median effective concentration
EDTA	Ethylenediaminetetraacetic acid
IGC_{50}	50 % impairment growth concentration
LDH	Lactate dehydrogenase
MIAN	Minimal information about nanomaterials
MNO	Manufactured nano-object
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
PCC	Physicochemical characterization
SEM	Scanning electron microscope
TEM	Transmission electron microscope

5 Materials

5.1 Test organism and culture medium

Tetrahymena is a genus of free-living ciliates, common in freshwater ponds and used as model organisms in biomedical research. There are different species of *Tetrahymena* sp. used as model organisms in biomedical research such as *T. thermophila* and *T. pyriformis*. Different species respond differently towards various toxicants because of differences in their uptake and metabolic processes. *Tetrahymena thermophila* is the more common species, which has been most commonly used in toxicity tests. This pear-shaped freshwater microorganism (30 µm × 50 µm) grows easily to high density in the laboratory.

Axenic cultures of *T. thermophila* from the Protoxkit FTM (MicroBioTests Inc.)[1)] grow for 24 h in the dark at 32 °C in a semi-defined proteose-peptone based medium[19] a nutrient rich medium (detailed information is provided in 8.2). The cell density obtained in these culture conditions is approximately 10^5 cells/cm^3. The cells are then processed according to method described by Schultz (1997)[19] in a nutrient poor medium. All experiments are performed in batch cultures of 100 cm^3 in Erlenmeyer flasks and aerated by shaking (90 rpm) in darkness.

5.2 Chemicals

5.2.1 General chemicals

— Potassium dichromate ($K_2Cr_2O_7$).

— Hydrogen peroxide (H_2O_2).

— Milli-Q water.

— DDW.

5.2.2 Additional chemicals for nutrient medium

— Proteose-peptone (bacteriological peptone).

1) Protoxkit FTM (MicroBioTests Inc.) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

— D-glucose ($C_6H_{12}O_6$).

— Yeast extract (for microbiology).

— Trizma-base ®[2] [Tris Hydroxymethyl Aminomethane Base, $(HOCH_2)_3CNH_2$].

— Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$).

— Copper(II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$).

— Iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$).

— Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$).

— Ammonium iron(II) sulfate hexahydrate ($Fe(NH_4)2(SO_4)_2{\cdot}6H_2O$).

— Magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$).

— Zinc chloride ($ZnCl_2$).

— EDTA.

— 37 % aqueous solution of hydrogen chloride (HCl).

— Cell proliferation kit I (MTT)[3].

— ATP bioluminescent assay kit.

— Trypan blue.

6 Technical equipment

— Adequate apparatus for temperature control.

— Light microscope equipped for imaging.

— Centrifuge.

— Pipettes.

— Laboratory oven.

— Autoclave.

— Sonicator (ultrasonic device).

— Plate stirrer.

— Spectrophotometer.

7 Preparation and characterization of the nano-object

7.1 Nano-object characterization

The complete physical-chemical characteristics of test nano-object (e.g. shape, purity, size) should be determined according to ISO/TR 13014. Particle morphology of test nano-object should be determined using TEM or SEM.

2) Trizma-base ® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

3) Cell Proliferation Kit I (MTT) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.