TECHNICAL SPECIFICATION

ISO/TS 4988

First edition
2022-05

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.

Reference number
ISO/TS 4988:2022(E)

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Published in Switzerland

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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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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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.

7.2 Dispersion preparation

The preparation of the MNO dispersion should be well documented, preferably via a standard operating procedure as this step in the testing is known to impact on the tested material. Dispersion is often done in a two-step procedure, first a stock suspension is prepared, and then an aliquot of this is further diluted when the testing starts. Dispersion of MNOs in stock suspension can be achieved by stirring or sonication at least 15 min (depends on type of MNOs) using ultrasonic device where appropriate. Stock suspensions shall be vortexed first and then sonicated for at least 15 min (depending on the type of MNO) using an ultrasonic device for the appropriate time. Sonication should be carried out in a way that produces no other new materials and the effects of sonication should be evaluated for each material. Reference [32] should be followed or use a list recommended as MIANs, which is designed to capture the "quality and quantity" of the physicochemical characterisation performed for that nanomaterial[25].

7.3 Dispersion characterization

Before each toxicity test, stock suspensions shall be vortexed and then sonicated for at least 15 min (depends on type of MNOs) using ultrasonic device for an appropriate time and diluted in the corresponding cell culture medium to the final concentrations. The suspensions should be characterised using TEM, DLS and zeta (ζ) potential measurements. The specimens for TEM analysis can be prepared by drying the aqueous suspension of MNOs at room temperature on a transparent carbon foil supported on a copper grid (or any other appropriate grid). Dispersion state of the MNOs can be characterized with DLS as described in ISO 22412 or other suitable methods such as ultrasonic attenuation spectroscopy as described in ISO 20998-1. Also, the concentration of the MNOs in the stock suspension should be evaluated using an appropriate method. In the case of nano-objects, which tend to release ions, dissolution should be assessed by an appropriate method. For example, in the case of metal-based nano-objects, AAS or ICP-MS can be used. The size distribution of the MNO dispersion and its stability over time shall be characterized before and after the exposure. As many characteristics of MNOs and MNOs in the suspension as possible should be evaluated according to the minimal information standard on MNOs characterization following descriptors of the Nanomaterial Registry MIANs' PCC[16]-[25].

7.4 Preparation of media for toxicity tests

All the exposure media are prepared fresh from stock solutions (see 8.2.2). Appropriate volumes of well mixed (homogenous) stock solutions should be added directly to Milli-Q water (see ISO 3639) with dispersed MNOs to achieve relevant concentrations of the MNO in exposure media.

8 Culture of *Tetrahymena* sp.

8.1 General

Tetrahymena was the first animal-like eukaryotic cell to be grown axenically (a culture in which only a single species is present) and can be easily cultured using a wide variety of media, containers and conditions, as long as basic requirements for nutrition, aeration, temperature and cell concentration are met. It is important to note that, regardless of the culture method employed, it is essential that care is taken to maintain cell viability when harvesting or manipulating the cells. *Tetrahymena* cells are extremely sensitive to the changes in aeration, cell concentrations and pressure resulting from centrifugal forces, much more so than bacteria or yeast[1].

8.2 *Tetrahymena* culturing conditions

8.2.1 *Tetrahymena* growth conditions

Axenic cultures of *Tetrahymena* sp. shall be grown for 24 h in the darkness at 32 °C in a nutrient rich medium which contains:

— 5 g D-glucose;

— 5 g proteose-peptone;

— 1 g yeast extract;

— 1,2 g Trizma-base ®;

— chlorides (2,28 µM $CaCl_2.2H_2O$, 0,29 µM $CuCl_2.2H_2O$, 0,05 µM $FeCl_3.6H_2O$, 0,03 µM $MnCl_2.6H_2O$, 0,004 µM $ZnCl_2$);

— sulphates (4,1 µM $MgSO_4.7H_2O$, 0,64 µM $Fe(NH_4)_2(SO_4)_2.6H_2O$);

— up to 1 000 cm^3 DDW;

— pH adjustment to 7,35 with HCl.

The cell density obtained in these culture conditions should be approximately 10^5 cells/cm^3 after 24 h.

NOTE 1 Cultured *Tetrahymena* sp. can be applied for using up to a one-week old culture as a 5 % inoculum.

NOTE 2 *T. thermophila* is difficult to store as it does not survive freezing. Therefore, it is necessary for it to be subcultured every week to two weeks.

8.2.2 *Tetrahymena* conditions during exposure

The cells are maintained in a nutrient poor medium (NPM) according to method described in Reference [19] during the experiment which contains:

— 5 g D-glucose;

— 1,2 g Trizma-base ®, 1 000 cm^3 DDW;

— pH adjusted to 7,4 with HCl;

— a temperature of 32 °C for the entire experiment.

9 Effect of nano-objects on *Tetrahymena* sp.

9.1 Test concentrations

9.1.1 Range finding test

If the toxicity of the test MNOs is not already known, a range finding test should be performed to determine the range of concentrations to be used in the definitive test. For a range finding test the *Tetrahymena* sp. should be exposed to a series of widely spaced concentrations of the test chemical (e.g. 0,01 mg/dm^3, 0,1 mg/dm^3, 1 mg/dm^3, 10 mg/dm^3, 100 mg/dm^3). *Tetrahymena* with a density of (10^5 cell/cm^3) should be exposed to each concentration of MNOs for a period of 24 h. The exposure period can be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. Three replicates are required and nominal concentrations of the chemical are acceptable.

9.1.2 Definitive test

The purpose of the definitive test is to determine the concentration-response curves and the 24 h EC_{50} values.

The dilution series spans the range of the lowest concentration producing 10 % effect and the highest one producing less than 100 % effect in the range finding test.

Tetrahymena should be exposed to five or more concentrations of the MNOs chosen in a geometric series in which the ratio is between 1,5 and 2,0 (e.g. 2 mg/dm^3, 4 mg/dm^3, 8 mg/dm^3, 16 mg/dm^3, 32 mg/dm^3, and 64 mg/dm^3). Equal densities of *Tetrahymena* should be placed in three or more replicates.

If solvents, solubilizing agents, or emulsifiers have to be used, they should not possess a synergistic or antagonistic effect on the toxicity test (they should be also applied in the control samples without MNOs).

The concentration of solvent should not exceed 100 mg/dm^3.

Test duration is 24 h. The test is unacceptable if more than 10 % of the control organisms are dead during the 24-h test period. Each test chamber should be checked for dead cells at 24 h after the beginning of the test. Concentration-response curves and 24 h or 48 h EC_{50} values for dead cells should be determined along with their 95 % confidence limits.

The test can be done in 96-well polystyrene plates or any other appropriate test vessel.

9.2 Duration

The effect of MNOs on *Tetrahymena* as well as the reaction of this organism to MNOs is directly related to the time of exposure. Generally, in the initial hours of exposure, cells are under stress conditions but after about one day (approximately 24 h) they can acclimate themselves to MNOs. The best exposure time should be 24 h.

9.3 Observations

Each test can be sampled every 2 h, when dynamics of a process is studied. In addition, any abnormal behaviour or appearance should be reported. It is possible to monitor different parameters that can explain the interactions or mechanisms of adaptations of *Tetrahymena* sp. to MNOs (such as particle phagocytosis, shape or behaviour of cells) but this is not really a core part of the growth inhibition assay. The cells can also respond to unfavourable conditions by forming cysts (but not very noticeable).

9.4 Detailed description of exposure condition

Since contamination can happen quickly, all the procedures should be performed using aseptic techniques and conditions (i.e. laminar flow hood). Incubation during exposure shall be in darkness; unless the light-associated effects of some MNOs are assessed (it is known that some MNOs can become more toxic when activated by light).

Temperature can be 25 °C to 32 °C and for each single test, it should be constant within ±1 °C and pH should be adjusted to 7,4. *Tetrahymena* should be in starvation condition during the test. Cells can be maintained in nutrient poor medium (in order to reduce the aggregation of MNO) for several days, although viability is decreased.

It is important to note that, regardless of the culture method employed, care shall be taken to maintain cell viability when harvesting or manipulating cells. The cells should be harvested using centrifugation at low speed (e.g. 3 min, 60 RCF), washed and then resuspended in a nutrient-poor medium before exposure to MNOs. *Tetrahymena* cells are extremely sensitive to the changes in aeration and cell concentration resulting from centrifugation, much more so than bacteria or yeast and it is important to consider the following points.

— It is essential for the supernate (centrifuged cells) to be removed immediately from the pellet since *Tetrahymena* are strong swimmers and significant numbers of cells can be lost from the pellet if the supernate is not swiftly removed.

— High centrifugal velocities, especially if accompanied by sudden, dramatic temperature changes, can cause massive cell lysis, as can too vigorous resuspension of cell pellets. Therefore, short centrifugation time in room temperature can be used.

At the beginning and end of the test, the total concentration of the test MNOs should be measured in at least the vessels that contain the highest and lowest test concentrations. Results should be based on measured concentrations. However, if evidence is available to demonstrate that the concentration

of the test MNOs has been satisfactorily maintained within ±20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

At the beginning and end of the test, the stability of the dispersion and the size distribution of the MNOs shall be verified in the highest and lowest test concentrations.

Every test should include controls consisting of the same dilution water, vehicle of nano-object, conditions and procedures, and *Tetrahymena* with same density.

Temperature and pH should be measured at the beginning and end of the test in each chamber.

9.5 Toxicity assessments

9.5.1 Cell viability

Total and dead cell numbers should be estimated by conventional direct microscopic counting using light microscope with a charge-coupled device (CCD) camera with a two hundred times magnification in a Neubauer chamber. A 10-mm^3 sample of culture should be transferred into a Neubauer chamber and dead cells stained with Trypan blue, including non-motile cells and cells with changed morphology can be counted, then 5 mm^3 of 4 % formalin should be added to kill the cells and a total cell count can be performed.

9.5.2 Population growth impairment tests

Population growth impairment assay is based upon population growth impairment. Since the optical density of the culture is related directly to the number of ciliates per unit volume, especially for log-growth or near log-growth phase, a spectrophotometric measure provides a rapid means of estimating population density. This short-term static assay uses population density quantified spectrophotometrically at 540 nm, with the endpoint recorded being the 50 % impairment growth concentration (IGC_{50}).

9.5.3 ATP assay

ATP bioluminescent assay kit can be employed for the quantitative bioluminescent determination of ATP. For ATP extraction, protozoa culture samples (100 mm^3) should be added to boiling extraction buffer (900 mm^3), 0,1 M Tris and 2 mM EDTA can be added and the pH should be up to 7,8 with HCl. The relative light units (RLU) should be recorded. The ATP concentration should be calculated using the formula:

$$C_{ATP} = 10^{(\log LUM - b)/a} * x$$

where

C_{ATP} is the concentration of ATP, in µmol/cm^3;

a is the constant of preportionality;

b is the initial value in the linear calibration curve;

x is the dilution factor.

9.5.4 MTT assay

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. Also, here the Cell proliferation kit I (MTT) can be used (see ISO 19007). This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells.

The effect of MNOs on cell viability can be detected via measurement of formazan absorbance (colour change).

In cases where MTT assay reagents interfere with tested particles, LDH assay can be used instead.

9.5.5 LDH assay

The LDH assay, also known as LDH release assay, is a cell death cytotoxicity assay used to assess the level of plasma membrane damage in a cell population. LDH is a stable enzyme, present in all cell types, which is rapidly released into the cell culture medium upon damage of the plasma membrane. LDH is the most widely used marker used to run a cytotoxicity assay.

The LDH assay protocol is based on an enzymatic coupling reaction: LDH released from the cell oxidizes lactate to generate NADH, which then reacts with WST reagent to generate a yellow colour. The intensity of the generated colour correlates directly with the number of lysed cells.

9.6 Phagocytic activity and material bioassimilation

The uptake of MNOs into food vacuoles should be studied from two perspectives including different times and different concentrations. Food vacuoles formed by phagocytosis (of test MNOs) can be counted oculometrically with light microscope. The percentage of cells containing at least one food vacuole filled with MNOs should be calculated as follows:

$V = C / T \times 100$

where

V is the vacuole formation", in percentage;

C is the number of cells containing at least one MNOs vacuole;

T is the total number of examined cells.

In addition, the modulation of phagocytic activity is investigated as an unconventional physiological stress/toxicity endpoint.

10 Data analysis

The EC_{50} can be calculated with several softwares, for example, EPA probit analysis program, PriProbit, SPSS, REGTOX software for Microsoft Excel™, etc and the EC_{50} values (effective concentration leading to a 50 % cell death) can be calculated with their 95 % confidence interval.

11 Test report

The test report shall include the following information.

a) Test nano-object:

- complete characterization of MNO and dispersion according to MIAN reporting standard for nano-objects;
- substance of test nano-object (manufacturer's code, catalogue or formulation number, batch number or date of manufacture, trade-name, etc.);
- all equipment and instrumentation used (manufacturer's model or catalogue number, serial number or date of manufacture, brand-name, etc.).

b) Test species: source and species of *Tetrahymena*, supplier of source (if known) and the culture conditions used (including source, kind and amount of substrate /medium).

c) Test conditions:

- description of test wells: type and volume of wells, volume of solution, number of *Tetrahymena* per test vessel, number of test wells (replicates) per concentration;
- methods of preparation of stock and test solutions, including the use of any solvents or dispersants, concentrations used;
- details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc.);
- incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc;
- the nominal test nano-object concentrations and the results of all analyses to determine the total concentration of the test nano-object during the test.

d) Bioassay results:

- percentage of those *Tetrahymena* that are coloured or show any adverse effects (including abnormal behaviour) in the controls and in each treatment group, at each observation time;
- percentage of MNO-filled vacuoles in each treatment group compared to the control, at each observation time;
- the calculated 24 h EC_{50} with the 95 % confidence limits;
- the data confirming the validity of the results:
  - mortality percentages of the controls;
  - explanation for any deviation from this document and whether the deviation affected the test results.

e) the International Standard used (including its year of publication);

f) any unusual features observed;

g) the date of the test.

12 Results validity with negative control

The test can be considered valid if the following conditions are fulfilled.

- The state of the control culture during the test period should be assessed by visualizing the cells under light microscope to observe the size, number and motility of the protozoa. Cells shall be mobile. Their density is determined spectroscopically.
- Cell number in the control culture can only be slightly reduced (maximum up to 15 % during 24 h), the cells remained active and motile. Thus, the results of the experiments can be considered reliable.
- The ATP content in the control culture should not be decreased more than 25 %.