PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Algal growth inhibition test with nanomaterials

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

Authored by:

Lars M. Skjolding, Sara N. Sørensen, Aiga Mackevica, Anders Baun

Technical University of Denmark, Denmark

Reviewed by:

Teresa Fernandes, Harriot-Watt University, United Kingdom

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1 Introduction:

DOMAIN: Exotoxicology

Standardized algal test guidelines developed by international organizations like International Organization for Standardization (ISO) and Organisation for Economic Co-operation and Development (OECD) as well as Standard Operating Procedures (SOPs) developed in a number of European Union (EU) projects prescribe that algal tests must be performed under stable test conditions in terms of pH, temperature, carbon dioxide levels and light intensity. This is crucial for inter- as well as intra-laboratory comparisons of test results and hence for the regulatory reliability of the test results. However, it is in practice not an easy task to maintain stable test conditions during a 72 hours algal test. Hence, algal tests results often suffer from poor reproducibility and reliability for a range of chemical substances and nanomaterials (NMs). In PATROLS, an increase in regulatory reliability of algal testing was addressed by improving the physical test system for algal tests. This led to the development of the LEVITATT testing platform (LED Vertical Illumination Table for Algal Toxicity Tests), documented in Skjolding et al. (2020).

Several test technical issues make it difficult to ensure optimal growth, low replicate variance, and uniform exposure conditions. Of these, the testing volumes, light conditions, and CO_2 exchange with the atmosphere are of high importance. Most of the existing algal toxicity testing setups operate with relatively large volumes (100-250 mL) situated on an orbital shaker inside an incubator. Such a setup limits the number of test concentrations and replicates achievable and requires high volumes of algal culture and test material. Additionally, these setups rarely have a uniform light field and reliable lighting conditions are furthermore difficult to obtain in large flasks, partly as light intensity decreases exponentially the further the light travels and partly due to the flask geometry.

Alternative setups comprise plastic microtiter plates containing small sample volumes that do not allow for adequate sampling volumes to measure pH, additional biomass measurements, pigment extraction or other analyses requiring destructive sampling.

One particular challenge using existing setups for algal toxicity testing of nanomaterials is the interference or blocking of the light available to the algal cells, often referred to as “shading”. Shading may occur within vials by the test material and/or interactions between test material and algal cells, or shading can occur between vials, due to their positioning relative to each other and the light source.

In order to overcome these challenges with the standard algal testing setups, the novel testing platform LEVITATT was developed. The testing platform and related SOP is based on the small-scale algal toxicity test setup introduced by Arensberg et al. (1995) that allows for testing in compliance with OECD TG 201 (OECD, 2011) as well the ISO 8692 test method (ISO, 1989). The method is further optimized to address the limitations stated above by; 1) utilizing LED light technology to ensure uniform light conditions with minimal heat generation, 2) providing adequate sample volume for chemical/biological analysis while maintaining constant pH, CO_2 levels, and 3) enabling the use of versatile test container material for testing of volatile substances or substances with a high sorption potential.

1.1 Scope and limits of the protocol

This procedure describes how to perform an algal growth inhibition test to determine the ecotoxicity of nanomaterials using the LEVITATT test setup in the framework of studies for the PATROLS project grant agreement no. 760813 under Horizon2020 research and innovation programme. LEVITATT setup provides a compact platform for algal toxicity testing of regular chemicals compliant with international standardized guidelines. Furthermore, the setup provides a robust platform for testing of difficult substances that interfere with the passage of light towards the algal cells e.g. nanomaterials.

Exponentially growing test organisms, *Raphidocelis subcapitata* (also referred to as *Selenastrum capricornutum*), are exposed in batch cultures to the NM dispersion over a period of 48 or 72 hours. The measured response is the reduction of growth rates in a series of algal cultures exposed to various concentration of NMs, compared of the average growth of unexposed control cultures. Growth and growth inhibition

are quantified as a function of time. The test endpoints are EC50 (effect concentration) and EC10 corresponding to a inhibition of growth rate by 50% and 10% respectively.

1.2 Validation state of protocol

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	x
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

If yes, specify

2 Terms and Definitions:

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 80004-1: 2016, definition 2.1]

Nanomaterial
Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Engineered nanomaterial
Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Manufactured nanomaterial
Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Substance
Single chemical element or compound, or a complex structure of compounds.

[SOURCE: ISO 10993-9:2009, definition 3.6]

3 Abbreviations:

EC_x - Effective Concentration

EDTA - (Ethylenedinitrilo)tetraacetic acid

EU – European Union

HPLC – High Performance Liquid Chromatography

ICP-MS – Inductively Coupled Plasma Mass Spectrometry

ICP-OES - Inductively Coupled Plasma Optical Emission Spectrometry

ISO – International Organization for Standardization

LEVITATT - LED Vertical Illumination Table for Algal Toxicity Tests

NM – Nanomaterial

NOEC - No Observed Effect Concentration

OECD - Organisation for Economic Co-operation and Development

SR-NOM - Natural Organic Matter from Suwannee River

4 Principle of the Method:

Exponentially growing test organisms, *Raphidocelis subcapitata* (also referred to as *Selenastrum capricornutum*), are exposed in batch cultures to the NM dispersion over a period of 48 or 72 hours. The measured response is the reduction of growth rates in a series of algal cultures exposed to various concentration of NMs, compared of the average growth of unexposed control cultures. Growth and growth inhibition are quantified as a function of time. The test endpoints are EC50 and EC10 corresponding to a inhibition of growth rate by 50 % and 10 % respectively.

5 Description of the Method:

5.1 Biological setting & test system used:

Exponentially growing test organisms, unicellular green algae species *Raphidocelis subcapitata* (also referred to as *Selenastrum capricornutum*) are used for the algae toxicity test described in this SOP. The culturing of these cells and prepaparation of algal growth medium to maintain the cell culture is described in detail under the Section 5.6 Procedure.

5.2 Chemicals and reagents used:

Chemical	CAS rn
HCl	7647-01-0
NaOH	1310-73-2
CH_3COCH_3 (Acetone): HPLC grade, saturated with magnesium carbonate	67-64-1
$K_2Cr_2O_7$	7778-50-9
NH_4Cl	12125-02-9
$MgCl_2 \cdot 6H_2O$	7791-18-6

$CaCl_2 \cdot 2H_2O$	10035-04-8
$MgSO_4 \cdot 7H_2O$	10034-99-8
KH_2PO_4	7778-77-0
$FeCl_3 \cdot 6H_2O$	10025-77-1
$Na_2EDTA \cdot 2H_2O$	6381-92-6
H_3BO_3	10043-35-3
$MnCl_2 \cdot 4H_2O$	13446-34-9
$ZnCl_2$	7646-85-7
$CoCl_2 \cdot 6H_2O$	7791-13-1
$CuCl_2 \cdot 2H_2O$	10125-13-0
$Na_2MoO_4 \cdot 2H_2O$	10102-40-6
$NaHCO_3$	144-55-8
Locust Bean Gum (Galactomannan polysaccharide) available from Sigma	-
Natural organic matter from Suwannee River (SR-NOM) available from the International Humic Substances Society (IHSS)	-

5.3 Apparatus and equipment used:

- Pipettes
- Spatulas
- 20 mL glass scintillation vials with plastic cap (e.g. from VWR) with a drilled hole for mass transfer of CO_2 from the atmosphere
- 50 mL glass measuring vials with stoppers
- 100 mL glass measuring vials with stoppers
- Analytical balance (4 digits)

- pH-meter
- LEVITATT testing platform – see decription in Skjolding et al. (2020)
- If the LEVITATT is not available, a shaking table kept in a temperature controlled room at 23±2 °C with constant illumination may be used. See details for light and shaking in OECD TG 201.
- Foil-wrapped screw-capped tubes of typical capacity 10 to 20 mL
- Fluorescence sceptrophotometer with an excitation wavelength of 430 nm and a measured emission wavelength of 671 ± 10 nm.
- In case of unavailability of a fluorescence measurements, a microplate reader may be used. Black microplates are necessary for fluorescence measurements and they must be made from an acetone resistant material. Polypropylene microplates from Greiner Bio One have demonstrated to be suitable for this purpose.
- Test organisms: the strain of *Raphidocelis subcapitata*

5.4 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

5.5 Reagent preparation:

5.5.1 Preparation of the algal growth medium

The algal growth medium is prepared by adding an appropriate volume of stock solutions 1-4 to sterile ultrapure water. The stock solutions of nutrients are prepared according to the Table 1.

Table 1: Concentrations of nutrients in solution for R. *subcapitata medium*

Stock solutions	Nutrient	Mass concentration in stock solution	Final mass concentration in test solution

1: Macronutrients	NH_4Cl	1.5 g/L	15 mg/L (N: 3.9 mg/L)
	$MgCl_2 \cdot 6H_2O$	1.2 g/L	12 mg/L (Mg: 2.9 mg/L)
	$CaCl_2 \cdot 2H_2O$	1.8 g/L	18 mg/L (Ca: 4.9 mg/L)
	$MgSO_4 \cdot 7H_2O$	1.5 g/L	15 mg/L (S: 1.95 mg/L)
	KH_2PO_4	0.16 g/L	1.6 mg/L (P: 0.36 mg/L)
2: Fe-EDTA[b]	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 µg/L (Fe: 13 µg/L)
	$Na_2EDTA \cdot 2H_2O$	100 mg/L	100 µg/L
3: Trace elements	H_3BO_3[a]	185 mg/L	185 µg/L (B: 32 µg/L)
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 µg/L (Mn: 115 µg/L)
	$ZnCl_2$	3 mg/L	3 µg/L (Zn: 1.4 µg/L)
	$CoCl_2 \cdot 6H_2O$	1.5 mg/L	1.5 µg/L (Co: 0.37 µg/L)
	$CuCl_2 \cdot 2H_2O$	0.01 mg/L	0.01 µg/L (Cu: 3.7 ng/L)
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 µg/L (Mo: 2.8 µg/L)
4: $NaHCO_3$	$NaHCO_3$	50 g/L	50 mg/L (C: 7.14 mg/L)

[a] H_3BO_3 can be dissolved by the addition of 0.1 M NaOH

[b] EDTA should be removed when testing metals, to avoid binding of metal ions

Sterilize the stock solutions by membrane filtration (mean pore diameter 0.2 µm) or by autoclaving (120 °C, 15 min). Do no autoclave stock solutions 2 and 4, but sterilize them by membrane filtration.

Store the solution in the dark at 4 °C.

<u>Procedure to make 1 L of algal growth medium:</u>

1. Transfer 500 mL sterilized ultrapure water into a 1 L sterilized 1 L volumetric flask and add an appropriate volume of the stock solution:
    - 10 mL of stock solution 1
    - 1 mL of stock solution 2
    - 1 mL of stock solution 3
    - 1 mL of stock solution 4
2. Fill up to 1000 mL with sterilized ultrapure water, stopper the flask and shake thoroughly to homogenize the algal growth medium

3. Before use, equilibrate the solution y leaving ti overnight in contact with air, or by bubbling with sterile, filtered air for 30 min. After equilibration, adjust the pH if necessary to 8.1±0.2, with either 1 M HCl or 1 M NaOH.

5.6 Procedure:

5.6.1 Culturing of the algae

All operation must be carried out under sterile conditions in order to avoid contamination with bacteria and other algae. *R. subcapitata* is generally easy to maintain in various culture media. Information on suitable media is available from the culture collections. The cells are normally solitary, and cell density measurements can easily be performed using an electronic particle counter or microscope.

Stock culture:

The stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes and transferred to fresh medium at least once every two months.

The stock cultures are grown in scintillation vials containing the appropriate medium (volume approximately 5 mL). When the algae are incubated in the LEVITATT, a weekly transfer is required. The growth rate of a species can be determined form the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture:

The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test (in growth medium) and used when still exponentially growing, normally after an incubation period of 2 to 4 days. When the algal culture contain deformed or abnormal cells, they must be discarded.

When the tests are conducted without EDTA in the growth medium (see Table 1), the pre-culture must also be EDTA-free.

5.6.2 Preparation of culture inoculum

The initial biomass must be the same in all test cultures and sufficiently low (e.g. $5 \cdot 10^3$ cells/mL to 10^4 cells/mL for 3 days pre-culturing) to allow exponential growth throughout the incubation period without risk of nutrient depletion. It should not exceed $3 \cdot 10^6$ cells/mL by the end of the test.

In order to adapt the algae to the test conditions and ensure that they are in the exponential growth phase when used to inoculate the test dispersions, a pre-culture is prepared 2-4 days before the start of the test. The algal biomass should be adjusted in order to allow exponential growth to prevail in the inoculum culture until the test starts.

The initial cell concentration recommended for *R. subcapitata* is $5 \cdot 10^3$ cells/mL for 72 hour tests. Taking into account the dilution of algal inoculum for the preparation of toxicant dilution series, a concentration inoculum of $5 \cdot 10^5$ cells/mL is required. It is of high importance to accurately determine the starting biomass since the testing result and variability will depend on this. Counting of algae by coulter counting, flowcytometer, or haemocytometer is recommended.

Measure the increase in biomass in the inoculum culture to ensure that growth is within the normal range for the test strain under the culturing conditions. To avoid synchronous cell divisions during the test a second propagation step of the inoculum culture may be required.

5.6.3 Preparation of test dilution

5.6.3.1 Preparation of aqueous dispersions of NMs

Preparation of aqueous disperision of NMs follow the OECD TG318 – Dispersion stability of nanomaterials in simulated environmental media (OECD, 2017) using the algal test medium (Table 2) as dispersion medium.

5.6.3.2 Replicates and controls

The test design includes a minimum of three replicates of each test concentration (three different dilution series from three different stock dispersions) for a statistically acceptable evaluation of algal growth inhibition. If determination of the NOEC (No Observed Effect Concentration) is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. The number of control replicates must be twice the number of replicates used for each test concentration, at least three. A separate set of test solution may be prepared for analytical determinations of test substance concentrations.

5.6.3.3 Preparation of dilution series

The concentration range in which effects are likely to occur may be determined on the basis of results from range-finding tests. For the final definitive test at least five concentration, arranged in a geometric series with a factor not exceeding 3.2, should be selected. The concentration series should preferable convert the range causing 5-75% inhibition of algal growth rate.

If the toxicity of the NMs to microalgae is approximately known, a definitive test can be performed immediately. If no information is available on its toxicity, two consecutive assays must be performed:

- A range finding test to determine the 0-100% tolerance range of the algae to the NM.

- A definitive test to determine with more precision the 50% inhibition threshold.

<u>Range finding test</u>

A “tenfold” dilution series must be prepared, starting at the concentration of NMs required as the first dilution level (C1).

4. Take five 50 mL measuring flasks and label them as follows: C1 – C2 – C3 – C4 – C5. As an example, Table 2 starts at 100 mg/L as the highest NPs concentration.

Table 2: Dilution series of NMs

Flask	NM concentration (mg/L)
C1	100 mg/L
C2	10 mg/L
C3	1 mg/L
C4	0.1 mg/L
C5	0.01 mg/L

5. Shake thoroughly the dispersion of NMs prepared in Section 5.6.3.1 and transfer the required volume into the C1 flask to prepare the first dilution. Add growth medium up to the 50 mL mark.
6. Transfer 45 mL growth medium into all the other flasks (C2 to C5).
7. Stopper flask C1 and shake thoroughly to homogenize the dispersions of NMs. Transfer 5 mL of the flask C1 into the flask C2, in order to prepare the second test concentration.
8. Repeat the operation indicated in step 7 for flasks C2 to C5, i.e.:
    - 5 mL from C2 to C3
    - 5 mL from C3 to C4
    - 5 mL from C4 to C5
9. Remove (and discard) 5 mL dispersion from flask C5. Then remove and discard 0.5 mL dispersion from each flask, in order to adjust the volume to prepare the appropriate algal concentrations.
10. Add 0.45 mL of the $5 \cdot 10^5$ cells/mL algal stock suspension to each flask, in order to obtain an initial concentration of $5 \cdot 10^3$ cells/mL. Stopper the flasks and shake them thoroughly to distribute the algal suspension evenly.
11. Add 5 mL of each suspension 20 mL scintillation vials marked according to concentrations e.g. C1A, C1B, C1C for three replicates of concentration C1.
12. Proceed to Section 5.6.4 Incubation.

<u>Definitive test</u>

The dilution series to be prepared spans the range of the lowest concentration producing 90-100% growth inhibition and the highest one producing 0-10% growth inhibition relative to the contril in the range finding test. The new concentration range to be tested will be named C1-C5.

4. Take five 50 mL measuring flasks and label them as follows: C1 – C2 – C3 – C4 – C5. C1 is the lowest concentration that produced 90-100% growth inhibition and C5 the highest that gave 0-10% growth inhibition in the range finding test.
5. Take one 100 mL measuring flask to make up 100 mL of the lowest concentration that produced 90-100% growth inhibition. Transfer the following volumes of this concentration from the 100 mL flask into the other flasks:
    - 50 mL to flask C1
    - 25 mL to flask C2
    - 12.5 mL to flask C3
    - 6.25 mL to flask C4
    - 3.125 mL to flask C5

6. Fill all 50 mL measuring flasks (C1 to C5) to the mark with growth medium
7. Remove (and discard) 0.5 mL dispersion from each flask, in order to adjust the volume to prepare the appropriate algal concentrations.
8. Add 0.5 mL of the $5 \cdot 10^5$ cells/mL algal stock suspension to each flask, in order to obtain an initial concentration of $5 \cdot 10^3$ cells/mL. Stopper the flasks and shake them thoroughly to distribute the algal suspension evenly.
9. Add 5 mL of each suspension 20 mL scintillation vials marked according to concentrations e.g. C1A, C1B, C1C for three replicates of concentration C1.
10. Starting from the concentration in flask C1, calculate the actual concentration of NMs in each flask (these figures will be needed for the endpoint estimation):
    - C1 = _____ mg/L
    - C2 = 0.50 · C1 = _____ mg/L
    - C3 = 0.25 · C1 = _____ mg/L
    - C4 = 0.125 · C1 = _____ mg/L

- C5 = 0.0625 · C1 = _____ mg/L

11. Proceed to Section 5.6.4 Incubation.

5.6.4 Incubation

The LEVITATT complies with requirements stated by ISO 8692. In short, incubation with continuous, uniform fluorescent illumination e.g. of “cool-white” or “daylight” type. The light intensity is within the range of 60-120 uE·m^{-2}·s^{-1} measured in the photosynthetically effective wavelength range of 400 - 700 nm using an appropriate receptor. An average light intensity within ± 15% is maintained over the incubation area. The flasks are incubated for 72 ± 2 h and a temperature of 23 ± 2 °C.

5.6.5 Measurements and analytical determinations

The algal biomass in each flask is determined every 24 hours during the test period. The small volumes removed from the test dispersion by pipette to make measurement should not be replaced. Measurement of algal growth is conducted by a algal pigment extraction procedure to determine the biomass concentration.

The test dispersions will be analyzed to verify the initial concentration and changes in exposure concentration during the test. At the start and end of the test, collect aliquots of the exposure dispersion and analyze them by the appropriate method (Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Inductively Coupled Optical Emission Spectrometry (ICP-OES)) to quantify the NM concentrations. Measure the pH of the dispersion at the beginning and at the end of the tests. The determination of test substance concentration can be performed on the three replicate flasks at each test concentration for statistical analysis. For unstable test substances, additional sampling for analysis at 24 hours intervals during the exposure period will be prepared in order to better define loss of the test substance during the test. Analysis of the concentration at the start and end of the test of a low and high test concentration and a concentration around the expected EC50 is sufficient when exposure concentration vary less than 20 % from nominal values

during the test. Analysis of all test concentration at the beginning and at the end of the test will be performed when concentration do not remain within 80-120 % of nominal. If analysis of the dispersed test substance concentration is required, it may be necessary to separate algae from the medium. Separation should preferable be made by filtration.

The algal growth inhibition test is a more dynamic test system than most other short-term aquatic toxicity tests. Consequently, the actual exposure concentration may be difficult to define, especially for adsorbing substances tested at low concentrations. In such cases, disappearance of the test substance from dispersion by adsorption to the increasing algal biomass does not mean that it is lost from the test system. When the results of the test is analyzed, it should be checked whether a decrease in concentration of the test substance in the course of the test is accompanied by a decrease in growth inhibition. To overcome this issue, aliquots of the exposure dispersion are collected and subjected to analysis before and after the algal filtration step to quantify the NM concentration.

At the end of the test, microscopic observation will be performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae, as may be caused by the exposure to the test substance.

5.6.5.1 Measurement of algal growth

The algal biomass in each flask is determined at 24, 48, 72 hours by fluorometric determinations of the pigment (mainly chlorophyll) concentrations. Extracted pigment is used as a means of deriving the biomass of an algal culture in the presence of NMs, which interfere with measurement of culture density normally made by optical absorbance. The particulates and cell debris settles to the bottom of the extraction tubes while the algal pigments are in solution and is measured fluorometrically.

Sampling and storage:

Algal pigments are sensitive to light and oxygen, especially when it is extracted. To avoid oxidative and photochemical destruction, the samples shall not be exposed to bright light or air.

Homogenization of the sample may in some cases increase the extraction efficiency.

Procedure:

Remove 0.4 mL of each culture into a sampling tube that fits the fluorometer that will be used for determination of the pigment concentration. To each tube add 1.6 mL acetone (saturated with magnesium carbonate). Cap the tubes and invert several times to mix. Place in a dark cupboard at room temperature for 1-7 days before determining the algal pigment concentration in a fluorescence spectrophotometer. Fluorescence is measured after 24 hours extraction at room temperature. NOTE: In case a fluorescence spectrophotometer is not available, a microplate reader could be used (see specifications in Section 5.3)

Acetone:

High Performance Liquid Chromatography (HPLC) grade, with magnesium carbonate (light powder) added. The $MgCO_3$ acts as a buffer to prevent low pH degrading the algal pigments, the presence of magnesium ions further protects against degradation by ensuring the magnesium which forms the central ligand of the chlorophyll tetramer is not stripped out. $MgCO_3$ is almost insoluble in acetone. Thus, it is only necessary to add enough to leave a visible deposit on the bottom of the bottle after settling. Resuspension of the acetone before using is not necessary but it does not affect the measurement either.

Fluoremetry:

Fluorescence is measured at room temperature in arbitrary units on a fluorescence spectrophotometer/microplate reader with an excitation wavelength of 430 nm and a measured emission wavelength of 671±10 nm. The signal should change less than 0.5 % per °C. Fluorescence figures are corrected for background fluorescence measured on solvents mixed with algal medium.

In order to obtain the algal biomass values from fluorescence measurements, standard calibration curves can be performed. Mayer et al. (1997) propose the following procedure: Obtain a single algal culture of $5 \cdot 10^5$ cells/mL and prepare a tenfold dilution series (range $5 \cdot 10^2$-$5 \cdot 10^5$ cells/mL) of each 10 mL. Extract three

replicates from each cell density to carry out the fluorescence measurements, and represent the corresponding standard curves (log cells/mL vs. log fluorescence). Averages of three replicate extracts must be corrected with background fluorescence of solvents mixed with growth medium, and standard deviation should not exceed 5%. To calculate the specific growth rate, prepare twelve control cultures of each 10 mL at an initial nominal density of 10^3 cells/mL. Incubate them for 3 days with daily sampling for fluorescence measurements. A straight line in the time-log (fluorescence) plot will indicate exponential growth with constant pimentl content; and low deviations among replicate cultures will indicate that both growth rate and algal pigment content are reproducible among replicates. To obtain this curve it is necessary to maintain constant and homogenous test conditions with regard to temperature and light, as both growth rate and pigment content vary considerably with light and temperature.

5.6.6 Testing for nanomaterial interference:

Shading caused by nanomaterials may interfere with the interpretation of results in algal growth rate inhibition tests. The decision-tree shown in Figure 1 provides specific advice on how to account for shading caused by nanomaterials in algal toxicity tests.

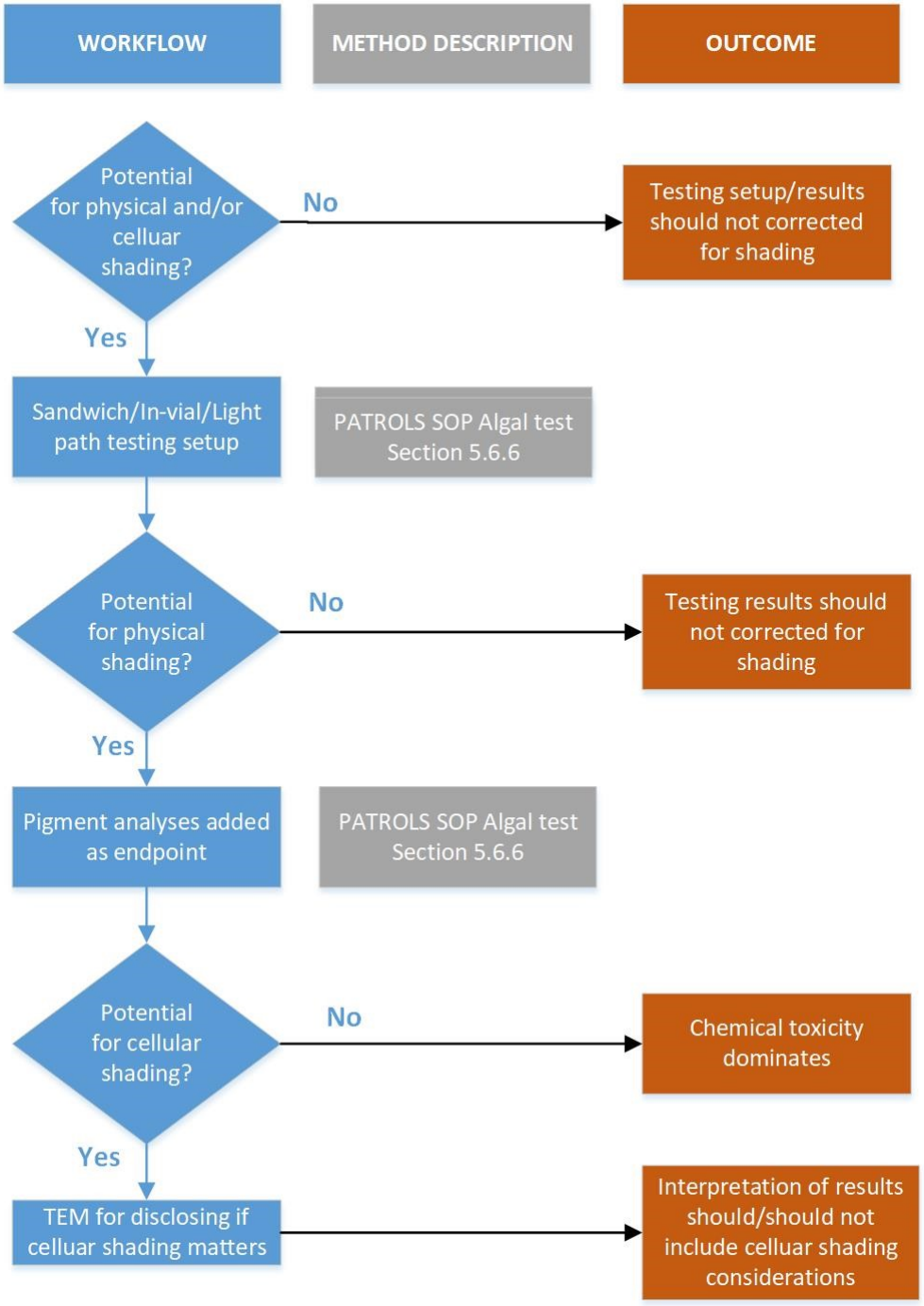

Figure 1. Decision tree for testing of the influence of shading when testing ENMs in algal toxicity tests.

The decision tree is divided in three parts: The work-flow for determination of the influence of shading on the testing outcome, the method description for the tests suggested, and the outcome of the testing.

To determine whether shading may occur, a visual inspection and/or a simple measurement of optical density of the highest test concentration is carried out. For a procedure for this, please see Skjolding et al. (2021).

If shading is considered to be a potential testing artefact, a test is carried out either with physical separation ("sandwich" or "in-vial" test) or preferably a test with shortened light path. If shading is found to influence the test result, a correction for this can be made provided that a concentration-dependent decrease in growth rates are found in the testing setups.

Results obtained in physical separation tests or tests with shortened light paths cannot alone reveal whether the growth rate inhibition is caused by physical and/or cellular shading. In this situation it is recommend supplementing these tests with testing of changes in algal pigment composition (see method decription below). If the analysis of changes in pigment content shows signs of shading, further TEM analysis can be performed to confirm that cellular shading plays a role.

Analysis of algal pigment composition

After 72 hours incubation, the algal suspensions in the nine vials per treatment were pooled to form triplicates, from which algal cells were collected on glass fiber filters (GF/A – 1.6 µm) by vacuum filtration. Each filter was folded to enclose the collected algal cells, packed in tin foil and stored at -80 °C until HPLC analysis. The filters containing algal cells were extracted in 3 ml 95% acetone with vitamin E acetate as an internal standard. The filters were sonicated in an ice-cooled sonication bath for 10 min, extracted further at 4°C for 20 h and mixed using a vortex mixer for 10 s. The filters and cell debris were filtered from the extracts into HPLC vials using disposable syringes and 0.2 µm Teflon syringe filters. Pigment analyses were carried out according to Schlüter et al. (2016) using the Van Heukelem & Thomas (2001) method, but with an adjusted pump gradient to optimize the pigment resolution. More than 30 different phytoplankton carotenoids and chlorophylls can be detected. The HPLC was a Shimadzu LC-10ADVP HPLC system composed of one pump (LC-10ADVP), a photodiode array detector (SPD-M10AVP), a SCL-10ADVP system

controller with Lab Solution software, a temperature-controlled auto sampler (SIL-10ADVP) (set at 4°C), a column oven (CTO-10ASVP), and a degasser (ERC 3415a). The HPLC system was calibrated using pigment standards from DHI Lab Products. Peak identities were routinely confirmed by online photo diode array analysis.

5.7 Quality control & acceptance criteria:

5.7.1 Validity criteria

1. The biomass in the control cultures should have increased exponentially by a factor corresponding to a specific growth rate higher than 0.9 day^{-1}. In tests performed with EDTA free growth medium this criterion is not applicable.
2. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3) in the control cultures must not exceed 35 %. This criterion applies to the mean value of coefficients of variation calculated for replicate control cultures.
3. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7 %.
4. The pH in the controls shall not have increased by more than 1.5 units relative to the initial pH in the growth medium. For metals and compounds that partly ionize at a pH around the test pH, it may be necessary to limit the pH drift to obtain reproducible and well defined results. A drift of < 0.5 pH units is technically feasible and can be achieved by ensuring an adequate CO_2 mass transfer rate from the surrounding air to the test dispersion, e.g. by increasing the shaking rate. Another possibility is to reduce the demand for CO_2 by reducing the initial biomass or the test duration.

5.7.2 Accuracy of the test

The SOP and the LEVITATT testing setup was tested in a round-robin test with five laboratories. The result for the round-robin test is shown in Table 3.

Table 3. Algal growth rate inhibition in terms of $EC_{50,72h}$ values, standard deviations on $EC_{50,72h}$, and coefficients variation of $EC_{50,72h}$ -values 3,5 dichlorophenol and CeO_2 nanoparticles (NM-212) tested in a round-robin test of the PATROLS SOP for algal toxicity testing and the LEVITATT testing setup.

Compound	Number of laboratories	$EC_{50,72h}$ (average) mg/L	Standard deviation	Coefficient of variation	Coefficient of variation reported in ISO 8692 round robin test (n=9)
3,5-Dichlorophenol	5	2.64	1.08	0.41	0.38
CeO_2 NP (NM-212)	4*	28.9	29.9	1.03	N/A
* One of the four participating laboratories used cell counting and not fluorescence for biomass deterimation. This may have resulted in a larger variability of the results (Hartmann et al., 2013)					

5.7.3 Reference test

It is recommend to used the compound 3,5 dichlorophenol as a reference toxicant in accordance with recommendations in ISO 8692. The $EC_{50,72h}$ should be comparable to the results obtained in the round-robin test of the SOP and the LEVITATT testing setup as shown in Table 3.

6 Data Analysis and Reporting of Data:

6.1 Plotting growth curves

A plot of growth curves can be made on a logarithmic scale using the relative biomass obtained from the fluorescence measurements. Generally, there is no need to convert the relative biomass back to number of algae cells. Exponential growth produces a straight line when plotted on a logarithmic scale, and the slope of the line indicate the specific growth rate. If procedural mistakes can be identified and/or considered higly likely, the specific data point is marked as an outlier and not included in subsequent statistical analysis e.g. zero algal concentration on one out of two or three replicate vessels may indicate the vessel was not inoculated correctly, or was improperly cleaned. State reasons for rejection of a data point as an outlier clearly in the test report. Accepted reasons are only (rare) procedural mistakes and

not just bad precision. Statistical procedures for outlier identification are of limited use for this type of problem and cannot replace expert judgement. Outliers should preferable be retained among the data points shown in any subsequent graphical or tabular data presentation for clarity.

6.2 Average growth rate

The growth rate is calculated as the logarithmic increase in biomass from the equation for each single flask of controls and NMs, equation (1):

$$\mu = \frac{(\ln(N_n) - \ln(N_0)}{t_d} \qquad (1)$$

Where μ is the growth rate, N_0 is the initial biomass (measurement of fluorescence at the beginning of test), N_n is the final biomass (measurement of fluorescence at the end of test) and t_d is the length of the test period in days.

The percent inhibition of growth rate can be calculated for each treatment replicate from equation (2):

$$\%Inhibiton = \frac{\mu_C - \mu_T}{\mu_C} * 100 \qquad (2)$$

Where, %Inhibition is the percent inhibition of growth rate, μ_C is the mean value for average growth rate in the control group and μ_T is the growth rate for each replicate of the test material.

If a dispersant is used, the dispersant controls rather than the controls without dispersant should be used in calculation of percent inhibition.

6.3 Plotting concentration response curve

Plot the percentage of inhibition against the logarithm of the test substance concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier in the first phase. Fit a line through the data points by computerized statistical method for interpolation. Depending on the intended usage of data, the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data

analysis at this stage and simply read the key figures EC50 and EC10 from the fitted curve. Valid reasons for not using a statistical method may include:

- Data are not appropriate for computerized methods to produce any more reliable results than can be obtained by expert judgment - in such situations some computer programs may even fail to produce a reliable solution (iterations may not converge, etc.)
- Stimulatory growth responses cannot be handled adequately using available computer programs.

6.4 Statistical procedures

For statistical processing a log-logistic function is fitted to the data using the R-script. The script will give EC10, EC20 and EC50 values with corresponding 95 % confidence intervals. The script also makes a plot visualizing the goodness of fit of the response data to the regression model.

Script for log-logistic function in R

The script uses the ratio for inhibition rather than the percentage and 2 degrees of freedom in order to fit datasets, which does not necessarily have a full concentration-response relationship.

Script for estimation of concentration-response relationships using a log-logistic function in R:

```
#Required library

library(tidyverse)

library(drc)

#Import your data or type your data dosea contains the concentrations used starting from the lowest to the highest and respa contains the response for the respective concentrations in relative %. Each replicate is separated by , and decimal separator is .

##Type your data

myd1 = tibble(
```

```
  dosea=c(0,0,0.93,0.93,2.34,2.4,5.85,5.85,14.6,14.6,36.5,36.5),
  respa=c(0,0,0.0025,0.043,0.047,0.12,0.03,0.08,0.17,0.15,0.46,0.44))#
#Check you data
myd1%>%head()
#plot your data with ggplot
myd1%>%ggplot(aes(x=dosea,y=respa))+
  geom_point()

#Plot your data
set.seed(100)
plot(drm(data = myd1,respa~dosea,fct=LL.2()),type="confidence",ylim = c(0,1))
plot(drm(data = myd1,respa~dosea,fct=LL.2()),type="all")
plot(drm(data = myd1,respa~dosea,fct=LL.2())),type="bars",ylim = c(0,1))
#Plot observed data and confidence interval of fit
plot(drm(data = myd1,respa~dosea,fct=LL.2()),type="confidence",ylim = c(0,1))
par(new=TRUE)
plot(drm(data = myd1,respa~dosea,fct=LL.2()),type="obs",ylim = c(0,1))
#Table for EC-values 10, 20, 50
EC <- drm(data = myd1, respa~dosea, fct=LL.2())
ED(EC, c(10,20,50), interval = "delta")
```

6.5 Growth stimulation

Growth stimulation (negative inhibition) at low concentrations is sometimes observed. This can result from either hormesis ("toxic stimulation") or from addition of stimulating growth factors with the test material to the minimal medium used. The addition of inorganic nutrients should not have any direct effect because the test medium should maintain a surplus of nutrients throughout the test. Low dose

stimulation can usually be ignored in EC50 calculations unless it is extreme. However, if it is extreme, or an ECX value for low x is to be calculated, special procedures may be needed. Deletion of stimulatory responses from the data analysis should be avoided if possible, and if available curve fitting software cannot accept minor stimulation, linear interpolation with bootstrapping can be used. If stimulation is extreme, use of a hormesis model may be considered, as specified in OECD 201 (OECD, 2011).

6.6 Test report

The test report must include the following:

1. Test substance: nanomaterial identification.
2. Test species: the strain, supplier or source and the culture conditions used.
3. Test conditions:
    - Date of start of the test and its duration.
    - Description of test design: test flasks, culture volumes, biomass density at the beginning of the test.
    - Test concentrations and replicates.
    - Description of the preparation of test dispersions, including use of dispersants.
    - Culturing apparatus.
    - Light intensity and quality (source, homogeneity).
    - Temperature.
    - Concentrations tested: the nominal test concentrations and any results of analysis to determine the concentration of the test substance in the test flasks. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported.
4. Results:
    - pH values at the beginning and at the end of the test at all test substances.
    - Biomass for each flask at each measuring point and method for measuring biomass.
    - Growth curves (plot of biomass versus time).

- Calculated response variables for each test replicate, with mean values and coefficient of variation for replicates.
- Graphical presentation of the concentration/effect relationship.
- Estimates of toxicity for response variables (EC50 and EC10) and associated confidence intervals.
- Any stimulation of growth found in any test substance.
- Any other observed effects, e.g. morphological changes of the algae.
- Discussion of the results, including any influence on the outcome of the test resulting from deviations from this procedure.

7 Publications:

Skjolding, L.M., Kruse, S., Sørensen, S.N., Hjorth, R. and Baun, A., 2020. A Small-Scale Setup for Algal Toxicity Testing of Nanomaterials and Other Difficult Substances. *Journal of Visualized Experiments: Jove*, (164).

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