INTERNATIONAL STANDARD

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# Water quality — Freshwater algal growth inhibition test with unicellular green algae

*Qualité de l'eau — Essai d'inhibition de la croissance des algues d'eau douce avec des algues vertes unicellulaires*



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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 8692 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 8692:1989), which has been technically revised.

# Water quality — Freshwater algal growth inhibition test with unicellular green algae

**WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.**



## 1 Scope

This International Standard specifies a method for the determination of the growth inhibition of unicellular green algae by substances and mixtures contained in water or by wastewater. This method is applicable for substances that are easily soluble in water.

With modifications to this method, as described in ISO 14442 and ISO 5667-16, the inhibitory effects of poorly soluble organic and inorganic materials, volatile compounds, heavy metals and waste water can be tested.

A rapid algal growth inhibition screening test for wastewater is included in Annex A.

## 2 Normative references

The following referenced documents are indispensable for the application 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 5667-16:1998, *Water — Sampling — Part 16: Guidance on biotesting of samples*

ISO 14442:1999, *Water quality — Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

**3.1**
**cell density**
$x$
number of cells per unit volume of medium

NOTE Cell density is expressed in cells per millilitre.

**3.2**
**specific growth rate**
$\mu$
proportional rate of increase in cell density per unit of time:

$$\mu = \frac{1}{x}\frac{\mathrm{d}x}{\mathrm{d}t}$$

where

$x$ is the cell density, expressed in cells per millilitre;

$t$ is the time, expressed in days

NOTE Specific growth rate is expressed in inverse days ($day^{-1}$).

**3.3**
**growth medium**
mixture of water and nutrients in which algal cells are incubated, which is used for pre-cultures and controls

**3.4**
**test sample**
aqueous sample (e.g. wastewater), chemical substance or mixture for which the inhibitory effects on the growth of algae are determined

**3.5**
**test medium**
mixture of water, nutrients and test sample

**3.6**
**test batch**
mixture of water, nutrients and test sample (test medium 3.5), inoculated with algae

**3.7**
**control**
mixture of water, nutrients (growth medium 3.3) without test sample, inoculated with algae

**3.8**
**effective concentration**
$E_rC_x$
concentration of test sample which results in a reduction of $x$ % in the specific growth rate relative to the controls

NOTE To unambiguously denote an EC value deriving from growth rate it is proposed to use the symbol "$E_rC$".

## 4 Principle

Monospecies algal strains are cultured for several generations in a defined medium containing a range of concentrations of the test sample, prepared by mixing appropriate quantities of growth medium, test sample and an inoculum of exponentially growing algal cells. The test batches are incubated for a period of (72 ± 2) h during which the cell density in each test solution is measured at least every 24 h.

Inhibition is measured as a reduction in growth rate, relative to control cultures grown under identical conditions.

## 5 Reagents and media

**5.1 Test organism**, using either of the following planktonic fresh water algae species:

a) *Desmodesmus subspicatus*[1)] (86.81 SAG).

b) *Pseudokirchneriella subcapitata* (Korshikov) Hindak[2)] (ATCC 22662, CCAP 278/4 or 61.81 SAG).

1) This species is formerly known as *Scenedesmus subspicatus* Chodat.

NOTE 1 Both algae species are planktonic green algae belonging to the order of *Chlorococcales* (*Chlorophyta, Chlorophyceae*), and are usually unicellular in culture.

The strains recommended are available in unialgal, non-axenic cultures from the following collections[3].

— SAG: Collection of Algal Cultures
Inst. Plant Physiology
University of Göttingen
Nikolausberger Weg 18
D-37073 Göttingen
Germany

— ATCC: American Type Culture Collection
12301 Parklane Drive
Rockville
Maryland 20852
USA

— CCAP: Culture Centre of Algae and Protozoa
Freshwater Biological Association
The Ferry House
Ambleside
Cumbria LA22 OLP
United Kingdom

Algothèque du laboratoire de cryptogamie
Muséum National d'Histoire Naturelle
12, rue Buffon
75005 Paris
France

NOTE 2 Stock cultures can be maintained in the medium described in 5.3. and 7.1. However, a frequent sub-culturing is necessary (once a week) to prevent failure of growth. The stock culture can be maintained for extended periods on richer algal media such as those recommended by the culture collection.

Alternatively algae can be stored for several months in alginate beads[4], without losing their viability[1]. The algae can be easily liberated from the algal beads when needed to perform the toxicity tests.

**5.2 Water**, deionized or of equivalent purity (conductivity < 10 µS/cm), for use in the preparation of the growth medium and test substance solutions.

Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage. Equipment made of copper shall not be used.

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2) This species is formerly known as *Selenastrum capricornutum* Prinz. The new name is currently cited by culture collections.

3) Trade name of strains are examples of suitable strains available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

4) The algae beads supplied by MICROBIOTESTS Inc., Venecoweg 19, 9810 Nazareth, Belgium. Tel. (32) 9 380 8545, fax (32) 9 380 8546, e-mail microbiotests@skynet.be, are an example of a suitable commercially available product. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

### 5.3 Nutrients.

Prepare four nutrient stock solutions in water, according to the compositions given in Table 1.

These solutions are eventually diluted (see 7.1 and 7.4) to achieve the final nutrient concentrations in the test solutions. However, the macro-nutrients may instead be added directly to the water.

All chemicals used shall be of reagent grade quality.

Sterilize the stock solutions by membrane filtration (mean pore diameter 0,2 µm) or by autoclaving (120 °C, 15 min). Store the solutions in the dark at 4 °C.

Do not autoclave stock solution 4 in order to avoid evaporative loss of $NaHCO_3$, but sterilize it by membrane filtration.

**Table 1 — Mass concentrations of nutrients in the test solution**

| Stock solution | Nutrient | Mass concentration in stock solution | Final mass concentration in test solution |
|---|---|---|---|
| Stock solution 1: macro-nutrients | $NH_4Cl$ | 1,5 g/l | 15 mg/l |
| | $MgCl_2 \cdot 6H_2O$ | 1,2 g/l | 12 mg/l |
| | $CaCl_2 \cdot 2H_2O$ | 1,8 g/l | 18 mg/l |
| | $MgSO_4 \cdot 7H_2O$ | 1,5 g/l | 15 mg/l |
| | $KH_2PO_4$ | 0,16 g/l | 1,6 mg/l |
| Stock solution 2: Fe-EDTA | $FeCl_3 \cdot 6H_2O$ | 64 mg/l | 64 µg/l |
| | $Na_2EDTA \cdot 2H_2O$ | 100 mg/l | 100 µg/l |
| Stock solution 3: trace elements | $H_3BO_3$[a] | 185 mg/l | 185 µg/l |
| | $MnCl_2 \cdot 4H_2O$ | 415 mg/l | 415 µg/l |
| | $ZnCl_2$ | 3 mg/l | 3 µg/l |
| | $CoCl_2 \cdot 6H_2O$ | 1,5 mg/l | 1,5 µg/l |
| | $CuCl_2 \cdot 2H_2O$ | 0,01 mg/l | 0,01 µg/l |
| | $Na_2MoO_4 \cdot 2H_2O$ | 7 mg/l | 7 µg/l |
| Stock solution 4: $NaHCO_3$ | $NaHCO_3$ | 50 g/l | 50 mg/l |

[a] $H_3BO_3$ can be dissolved by the addition of 0,1 mol/l NaOH.

## 6 Apparatus

All equipment that comes in contact with the test medium shall be made of glass or other chemically inert material.

Standard laboratory apparatus and the following.

**6.1 Temperature-controlled cabinet or room**, with a white fluorescent light, providing continuous, uniform illumination suitable for the lighting requirements as specified for the test in 7.6.

**6.2 Apparatus for measuring algal cell density**, preferably a particle counter, or a microscope and a counting chamber. Alternatively the algal densities may be determined by an indirect procedure using for instance a fluorimeter (e.g. *in vitro* fluorescence[2] or DCMU [5])-enhanced *in vivo* fluorescence[3]), when sufficiently sensitive and if shown to be sufficiently well correlated with cell density. The apparatus used shall be capable of measuring cell densities as low as $10^4$ cells/ml and to distinguish between algal growth and disturbing effects, for example the presence of particulate matter and the colour of the sample. Spectrophotometers may be sufficiently sensitive to measure $10^4$ cells/ml providing a sufficient path length (up to 10 cm) can be used. However, this technique is particularly sensitive to interferences from suspended material and coloured substances at low cell densities.

**6.3 Culture flasks**, for example 250 ml conical flasks with air permeable stoppers.

**6.4 Apparatus for membrane filtration**, using filters of mean pore diameter 0,2 µm.

**6.5 Autoclave**.

**6.6 pH meter**.

## 7 Procedure

### 7.1 Preparation of growth medium

Prepare a growth medium by adding an appropriate volume of the nutrient stock solutions (5.3) to water.

Add to approximately 500 ml of water:

— 10 ml of stock solution 1 (5.3);

— 1 ml of stock solution 2 (5.3);

— 1 ml of stock solution 3 (5.3);

— 1 ml of stock solution 4 (5.3).

Make up to 1 000 ml with water.

When autoclaving is necessary, stock solution 4 should be added afterwards.

Before use, equilibrate it by leaving overnight in contact with air, or by bubbling filtered air through it for 30 min. After equilibration, adjust the pH if necessary to 8,1 ± 0,2, using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

This growth medium is buffered by bicarbonate and atmospheric $CO_2$. Different pH values may be obtained by modifying the concentration of $HCO_3$ and/or the atmospheric $CO_2$ concentration (requires closed vessels) as specified in ISO 14442. Should such modifications be required in order to perform a test at a different, specific pH value, these should be clearly motivated and reported.

### 7.2 Preparation of pre-culture and inoculum

A pre-culture shall be started two to four days before the beginning of the test. Growth medium (7.1) is inoculated at a sufficiently low cell density (e.g. $5 \times 10^3$ cells/ml to $10^4$ cells/ml for three days pre-culturing) in order to maintain exponential growth until test start. The pre-culture shall be incubated under the same conditions as those in the test (7.6).

5) DCMU = dichlorophenyldimethyl urea (CAS No. 330-54-1).

This exponentially growing pre-culture is used as an inoculum for the test. Measure the cell density in the pre-culture immediately before use in order to calculate the required inoculum volume.

### 7.3 Choice of test sample concentrations

Algae should be exposed to concentrations of the test sample in a geometric series with a ratio not exceeding 3,2 (e.g. 1,0 mg/l, 1,8 mg/l, 3,2 mg/l, 5,6 mg/l and 10 mg/l).

The concentrations should be chosen to obtain at least one inhibition below and one inhibition above the intended $E_rC_x$ parameter. Additionally, at least two levels of inhibition between 10 % and 90 % should be included in order to provide data for regression analysis.

A limit test with only one concentration can be conducted to demonstrate absence of toxicity. The number of replicates for this one concentration should be six.

NOTE A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of difference in test concentration. Replication of test concentrations is not a requirement in the preliminary test.

### 7.4 Preparation of test sample and stock solutions

In case the test sample is aqueous (e.g. wastewater), pre-treatment (e.g. filtration, neutralisation) should be considered, depending of the nature of the sample and the purpose of the test. Add nutrient stock solutions (5.3) as described in 7.1 to the sample.

For non-aqueous test samples, preparation of stock solutions is generally necessary. The method for preparation of the stock solutions should be carefully chosen, based on the properties of the sample. Stock solutions are normally prepared by dissolving the test sample in growth medium. Modifications are necessary when the test sample does not readily dissolve in the test medium as specified in ISO 14442 and ISO 5667-16.

Normally, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution (see ISO 5667-16).

### 7.5 Preparation of test and control batches

Prepare the test and control batches by mixing the appropriate volumes of test sample or test sample stock solutions growth medium and inoculum (7.2) in the test vessels. The total volume, concentrations of added growth medium nutrients and cell density shall be the same in all vessels.

The initial cell density shall be sufficiently low to allow exponential growth in the control culture throughout the test duration without a pH drift of more than 1,5 pH units (see Clause 8). Therefore the initial cell densities shall not exceed $10^4$ cells/ml.

Prepare at least three replicates for each test sample concentration. To six further vessels, add only culture medium and inoculum with no test sample. These vessels serve as controls. If appropriate, prepare a single concentration series of the test sample without algae to serve as background for the cell density determinations.

The number of replicates per concentration might be reduced based on statistical considerations (see ISO/TS 20281), if increasing the number of concentrations and reducing the concentration spacing.

Measure the pH of a sample of each test batch and of the controls.

### 7.6 Incubation

The test vessels shall be sufficiently covered to avoid airborne contamination and to reduce water evaporation, but they shall not be airtight in order to allow $CO_2$ to enter the vessels (a small hole is sufficient). Incubate the test vessels at 23 °C ± 2 °C, under continuous, white light. The light intensity at the average level of the test media shall be homogenous within ± 10 % and in the range 60 µmol/($m^2$·s) to 120 µmol/($m^2$·s) when measured in the photosynthetically effective wavelength range of 400 nm to 700 nm, using an appropriate receptor.

It is important to note that the method of measurement, in particular the type of receptor (collector), affects the measured value. Spherical receptors (which respond to light from all angles above and below the plane of measurement) and "cosine" receptors (which respond to light from all angles above the measurement plane) are preferred to unidirectional receptors. They give higher readings for a multi-point light source of the type described in the Note.

NOTE The light intensity specified above can be obtained using four to six fluorescent lamps of the universal white (natural) type {i.e. a rated colour of standard colour 2 (a colour temperature of 4 300 K) according to [4]}. The optimum distance of the lamps is approximately 0,35 m from the algal culture medium.

For light-measuring instruments calibrated in lux, an equivalent range of 6 000 lx to 10 000 lx is acceptable for the test.

Testing of coloured test solutions requires specific modifications as described in ISO 14442.

Continuously shake, stir or aerate the cultures in order to keep the cells in free suspension and to facilitate $CO_2$ mass transfer from air to water, and in turn reduce pH drift.

### 7.7 Measurements

Measure the cell density in each test vessel (including the controls) at least every 24 h. Aliquots removed from the test batches for measurements should preferably not be replaced.

The nominal cell density can be used as the initial cell density and no initial cell density measurement is then required.

The test shall last for 72 h ± 2 h.

At the end of the test, measure the pH of samples of each test batch (7.5) and of the controls (7.5). The appearance of the cells and the identity of the test organism should be confirmed by microscopy.

## 8 Validity criteria

Consider the test invalid if the following conditions are not met.

a) The average control growth rate shall be at least 1,4 $d^{-1}$. This growth rate corresponds to an increase in cell density by a factor 67 in 72 h.

b) The variation coefficient of the control growth rates shall not exceed 5 %.

c) The control pH shall not have increased during the test by more than 1,5 relative to the pH of the growth medium.

An increase in pH during the test can have significant influence on the results and therefore a limit of 1,5 units is set. These variations, however, should always be kept as low as achievable, e.g. by performing continuous shaking during the test.

If these criteria are not met, examine experimental techniques and use inocula from other sources, if necessary.

# 9 Calculation

## 9.1 Plotting of growth curves

Tabulate the cell density measurements or other parameters correlated with cell density in the test media according to the concentration of test sample and the time of measurement.

Plot a growth curve for each test concentration and control, as a graph of the logarithm of the mean cell density against time. A linear growth curve indicates exponential growth, whereas a levelling off indicates that cultures entered the stationary phase.

If the control cultures show declining growth rate towards the end of the exposure period, inhibited cultures may tend to catch up with the controls, falsely indicating a decreased growth inhibiting effect. In this case, perform the calculations of growth rate and growth inhibition based on the last measurement within the exponential growth period in the control cultures.

## 9.2 Calculation of percentage inhibition

First calculate the average specific growth rates, $\mu$, for each test culture, using Equation (1).

$$\mu = \frac{\ln x_L - \ln x_0}{t_L - t_0} \quad (1)$$

where

$t_0$ is the time of test start;

$t_L$ is the time of test termination [or the time of the last measurement within the exponential growth period in the control cultures (9.1)];

$x_0$ is the nominal initial cell density;

$x_L$ is the measured cell density at time $t_L$.

Alternatively determine the average growth rate from the slope of the regression line in a plot of the natural logarithm of the mean cell density against time (9.1).

Calculate the mean value of $\mu$ for each test and control batch replicate. From these values, calculate the percentage inhibition for each test batch replicate, from Equation (2).

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100 \quad (2)$$

where

$I_{\mu i}$ is the percentage inhibition (growth rate) for test concentration $i$;

$\mu_i$ is the mean growth rate for test concentration $i$;

$\mu_c$ is the mean growth rate for the control.

### 9.3 Determination of $E_rC_x$ (e.g. $E_rC_{10}$ and $E_rC_{50}$)

Tabulate and plot for each individual flask the normalised inhibition ($I_{\mu i}$) against the test concentration on a logarithmic scale. If the scatter of data points is large, plot means of replicates with corresponding standard deviations.

Fit a suited non-linear model to the experimental data points by regression analysis (for example see ISO/TS 20281, References [9] and [10]) in order to determine $E_rC_x$ values, preferably with their confidence intervals.

If data are too few or uncertain for regression analysis, or if inhibitions appear not to follow a regular concentration response relation (e.g. stimulation), then a graphical method might be applied. In this case, draw a smooth eye fitted curve of the concentration response relationship and read $E_rC_x$ values from this graph. If extreme stimulation at intermediate concentrations of the test substance is observed, use of a hormesis model should be considered[8].

## 10 Expression of results

Denote $EC_{10}$ and $EC_{50}$ values based on growth rate as $E_rC_{10}$ and $E_rC_{50}$. Also indicate clearly the time span used for the determination, e.g. $E_rC_{50}$ (0 to 72 h). Quote $E_rC_{10}$ and $E_rC_{50}$, normally in mg/l or ml/l and the corresponding confidence intervals.

Where testing wastewater by means of a graduated dilution ($D$), the test medium with the highest concentration at which an inhibition < 5 % is observed, is termed the “lowest ineffective dilution (LID)”. This dilution is expressed as the reciprocal value of the volume fraction of wastewater in the test medium [e.g. if the wastewater content is one part in four (25 % volume fraction), the dilution factor is $D$ = 4]; see Annex A of ISO 5667-16.

## 11 Interpretation of results

$EC_{10}$ and $EC_{50}$ values are toxicological data derived from a laboratory experiment carried out under defined standard conditions. They give an indication of potential hazard, but cannot be used directly to predict effects in the natural environment.

When interpreting $EC_{10}$ and $EC_{50}$, take into consideration the shape of the growth curves. Certain features of these curves (e.g. delayed onset growth, good initial growth but not sustained) may help to indicate the mode of action of the toxic substance concerned.

## 12 Precision

Interlaboratory tests based on the test described in this International Standard were carried out in 1980 and 1981 (see References [9] and [10]). The results obtained with the reference substances $K_2Cr_2O_7$ and 3,5-dichlorophenol are shown in Table 2. Review of reference tests indicated that the sensitivity of the strains have not changed significantly for years.

**Table 2 — Interlaboratory test results for $E_rC_{50}$**

| Test organism and test substance | Number of laboratories<br>*l* | Outliers<br>*o* | Mean value<br>mg/l | Standard deviation<br>mg/l | Coefficient of variation<br>% |
|---|---|---|---|---|---|
| *Desmodesmus subspicatus* | | | | | |
| Potassium dichromate | 20 | 4 | 0,84 | 0,12 | 14 |
| 3,5-Dichlorophenol | 18 | 2 | 6,42 | 2,38 | 37 |
| *Pseudokirchneriella subcapitata* | | | | | |
| Potassium dichromate | 9 [a] | 4 | 1,19 | 0,27 | 23 |
| 3,5-Dichlorophenol | 9 [a] | 4 | 3,38 | 1,30 | 38 |

[a] The high number of outliers in the tests with *Pseudokirchneriella subcapitata* is due to the use of different growth media (with different pH-values). Results from tests with media with pH deviating from the growth medium specified in this International Standard have been excluded.

To prove the validity of the test system it is recommended to test at least one reference substance (e.g. when using a strain or after changing test conditions). Results should be compared to those given in Table 2.

NOTE The mean control growth rates determined in the inter-laboratory test were 1,74 $d^{-1}$ [coefficient of variation (CV 27 %)] for *Desmodesmus subspicatus* and 1,91 $d^{-1}$ (CV 23 %) for *Pseudokirchneriella subcapitata*. These growth rates suggest an increase in cell density by at least 150 cells/ml.

## 13 Test report

The test report shall include the following information:

a) a reference to this International Standard (ISO 8692:2004);

b) all data required for identification of the test sample;

c) test organism: species, origin, strain number, method of cultivation;

d) test details:

- start date and duration;
- method of preparation of sample and test batches;
- concentrations tested;
- composition of medium;
- culturing apparatus and incubation procedure;
- light intensity and quality;
- temperature;
- pH of test solutions including the controls at start and end of test;
- method for measuring cell density and, if appropriate, method to correct for background values;

e) results:

— cell density in each flask at each measuring point;

— mean cell density for each test concentration (and control) at each measuring point;

— growth curves (logarithm of cell density against time);

— relationship between the concentration and effect (percentage inhibition values against concentration) in table or graphical representation, e.g. percentage of inhibition on probit scaled ordinate against concentration in logarithmic scaled abscissa;

— $E_rC_x$ values such as $E_rC_{10}$ and $E_rC_{50}$ including the method of determination;

— other observed effects such as bleaching of algal cells.

# Annex A
(informative)

# Rapid screening of wastewater algal growth inhibition

## A.1 General

This International Standard can be applied to testing of effluents, wastewaters and other environmental aqueous samples. The following modifications address mainly requirements for carrying out screening tests in various types of test containers, e.g. microplates.

## A.2 Sampling and storage

Samples need to be tested as soon as possible after collection or occasionally frozen and thawed, filtered or centrifuged. The recommendations of ISO 14442 and ISO 5667-16 should be considered before planning wastewater testing.

## A.3 Culture vessels

Flasks, vials or microplates with appropriate culture volumes are used. The material and geometry of the test containers shall be chosen to avoid:

a) release of potentially toxic substances;

b) adsorption of components from the test media;

c) evaporative losses of important wastewater constituents;

d) light inhomogeneities among replicates and treatments.

## A.4 Choice of test concentrations

Prepare a dilution series of the water sample described in A.5. The dilution series should follow a geometric progression covering the desired range of response. If microplates or automated systems are used, an increase of number of tested dilutions is recommended in order to ensure compliance with this requirement. A range-finding test may be carried out to define the dilution series.

Use at least three replicates per treatment (including a control) and five concentrations unless there is sufficient technical justification for a different test design. The number of replicates per concentration can be reduced based on statistical considerations, by increasing the number of concentrations and reducing the concentration spacing.

## A.5 Preparation of test batches

Prepare the series of test batches in a manner that ensures that all batches receive the same concentrations of spiked nutrients and algal inoculum. This can for example be achieved by spiking the test water sample with nutrient stock solutions (5.3) as described in 7.1 and mixing the spiked water sample with appropriate volumes of growth medium and inoculum culture. This protocol allows testing concentrations up to approximately 98 %.