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

Qualité de l'eau — Lignes directrices pour essais d'inhibition de la croissance algale avec des matières peu solubles, des composés volatils, des métaux et des eaux résiduaires

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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 14442 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 14442:1999), which has been technically revised.

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

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.

IMPORTANT — It is absolutely essential that tests conducted according to this standard be carried out by suitably trained staff.

1 Scope

This International Standard provides procedures, not covered by the methods described in ISO 8692 and ISO 10253, for testing difficult substances for inhibition of algal growth.

The main subjects covered by the guideline are the methods for preparing the test substance for testing and the procedures needed to carry out an appropriate test. The following test substances are covered by this guideline:

a) poorly soluble pure organic compounds;

b) poorly soluble mixtures of organic substances;

c) poorly soluble inorganic materials;

d) volatile substances;

e) waste waters and environmental samples containing water and sediments;

f) coloured and/or turbid samples;

g) compounds of heavy metals.

The following methods of addition are covered:

— direct;

— dispersion;

— water-soluble and water-accommodated fractions.

Some guidelines related to the analytical procedures and to the interpretation of the results have been included.

References to documents describing the background for the testing of difficult substances are given in the Bibliography.

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, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 8692, *Water quality — Freshwater algal growth inhibition test with unicellular green algae*

ISO 10253, *Water quality — Marine algal growth inhibition test with* Skeletonema costatum *and* Phaeodactylum tricornutum

3 Analytical characterization of test materials and confirmation of concentrations and stability

Analytical characterization of test substances and materials and the confirmation of their concentrations and stability in the testing environment is of major concern of regulatory authorities. Such activities are usually not an integral part of this International Standard algal growth inhibition test methods.

However, there may be situations where analysis may assist in defining the appropriate exposure conditions of test materials and chemicals and/or in the interpretation of the results.

The relevant properties of substances and materials can be assessed from basic properties such as solubility in water, partition coefficient (lg P_{ow}), Henry's constant, photochemical and hydrolytic stability and biodegradability.

Analytical confirmation is strongly recommended in order to confirm test substance concentrations and is required for the calculation of effective concentration (EC) values of volatile substances (Clause 7). If losses due to adsorption on the test vessels or during transfer of test solutions and media occur, then analytical confirmation are of particular importance. This aspect is also specified in ISO 5667-16.

Due to the batch test system used for algal growth inhibition tests, loss of substances due to biodegradation (nearly all algal cultures contain bacteria), photodegradation, hydrolysis and/or adsorption cannot always be avoided. A decrease in measured concentrations is difficult to prevent by technical means, and is therefore considered acceptable for algal growth inhibition tests.

The following precautions are suggested for maintaining test substance concentrations in algal growth inhibition tests:

a) sterilization of media and equipment to reduce the effect of bacterial growth;

b) change of the light quality to prevent photodegradation of test substances;

c) avoidance of contact of test substance with water prior to testing to reduce hydrolytic decomposition;

d) treatment of glassware (e.g. silanization); the effectiveness of such a treatment varies from one chemical to the other;

e) pre-conditioning of the glassware, before addition of the test media, with the test substance at concentrations to be used in the test.

The effect of such technical measures is, if relevant and if possible, monitored by chemical analysis.

Water, waste water and organic/inorganic solids/liquids may contain components that may modify the composition of the algal growth medium (by precipitation of a limiting nutrient, complexation of essential elements, addition of nutrients), and subsequently may cause effects on algal growth not related to toxic

components. If such problems occur, it may be advisable to determine the content of key components of the test material. Some relevant components are: calcium, magnesium, sodium, potassium, sulfate, chloride, ammonium, nitrate, phosphate, copper, cobalt, nickel, zinc, cadmium, organic matter [i.e. measured as Chemical Oxygen Demand (COD) and/or Total Organic Carbon (TOC)].

If the material contains a high concentration of readily degradable organic material, the subsequent bacterial growth may disturb the algal growth measurement. When untreated (not filtered or centrifuged) waste water is tested, contamination with other algal species may occur.

4 Poorly soluble organic substances

4.1 General

A pure substance is a substance with one major component containing minor components as impurities. Poorly soluble substances are those with solubility limits below 100 mg/l in water. If, however, growth inhibition occurs at concentrations much lower than the solubility limits in water or algal growth medium (the limit in the medium may be different), then the poorly soluble substance can be tested as a water soluble substance (added via a stock solution in test medium). This approach is usually not applicable to substances with a water solubility below 1 mg/l to 10 mg/l (substances with a very low solubility).

The methods described in this clause therefore refer to testing of substances causing effects on algal growth at concentrations at or around the solubility limit in water and to very low solubility substances.

Testing of nominal concentrations markedly above the solubility limit is not recommended. It may, however, be unavoidable if the solubility limit in water or algal growth medium (which may be different) is not well established, or if a substance spontaneously forms dispersions in the test medium.

NOTE Terminology according to Reference [3]:

— water solubility below 100 mg/l: “sparingly soluble”;

— water solubility below 1 mg/l: “very low solubility”.

A number of methods which are available for preparing test solutions of pure substances, are described in ISO 5667-16. Generally, it is preferred to use mechanical means to prepare stock solutions.

4.2 Preparation of saturated and supersaturated solutions

If the solubility of a substance in water is between 1 mg/l and 100 mg/l, saturated solutions can be prepared by direct addition of the test substance. A saturated solution is usually prepared by stirring (e.g. magnetic stirrer or shaking, see also 5.1) an excess amount of the test substance in water for a period in test medium. A period of 20 h is practical for most substances, but a stirring period of up to three days may be considered to ensure saturation provided the substance is stable. Lengthy stirring should be carried out in the dark and in the same temperature range as the growth inhibition test is carried out. Preferably, the equilibrium should be confirmed by chemical analysis. After a phase separation period of varying length, the clear phase is collected and tested as the highest concentration. Filtration (through a 0,45 µm membrane filter) or centrifugation may be useful for removing particulate matter.

Certain membrane filters may interfere with the test substance. The type of filter should be chosen according to the physico-chemical properties of the test substance and the recommendations of the filter supplier.

Further test concentrations can be prepared by dilution of the saturated solution with test medium. A small volume of a concentrated suspension of algal culture is then added to the test media to start the test.

A disadvantage of preparing saturated solutions in this way is that trace impurities in the test substance may be preferentially enhanced in the solution, if they are more soluble than the major component. For this reason, the quantity of test substance should be the minimum required ensuring that a saturated solution of the test substance can be achieved.

Where possible, prepare stable supersaturated stock solutions with a substance (i.e. stock solution concentrations in the range 2 to 10 times the saturation value) in test medium by high speed mechanical stirring [e.g. a high speed blender [1)]] or ultrasonic treatment (a recommended frequency of 20 kHz and a power output of at least 60 W) for a few minutes to several hours. With both methods, a constant temperature shall be maintained during the treatment by cooling. If phase separation takes place immediately after the treatment has ended, one may choose to remove (sinking or floating) particles by filtration through a paper filter [e.g. Schleicher & Schüll 604 [2)]] or by centrifugation. If dissolved substances are removed by filtration, it is essential to confirm the actual concentrations in the final solution by chemical analysis. The test solutions can be prepared by dilution of the supersaturated stock solution.

4.3 Solvent addition

The use of a solvent as a carrier to add a substance to a test medium is considered to be a practical and convenient method for handling organic substances tested at concentrations below 10 mg/l. The recommended concentration of solvent does not influence the solubility of substances but assists in a rapid and complete mixing of substances and test medium.

At concentrations of the test substance below 1 mg/l the solvent addition may be combined with the methods described in 4.2 to prepare saturated solutions (which are further treated as described in 4.2).

In principle, any organic solvent can be used that meets the following criteria:

a) does not inhibit the algal growth at the highest concentration added;

b) is soluble in water at the recommended concentration;

c) does not interact with medium components;

d) does not react with the test substance;

e) does not biodegrade rapidly;

f) does not interfere with the conditions of illumination.

The concentration of a solvent should not exceed 100 µl/l test medium according to ISO 10253 and ISO 8692. In practice, this solvent concentration for algal tests can be obtained by the addition of 10 µl solvent per 100 ml of test medium, a solvent volume that can be added with precision. Solvents such as acetone and *t*-butanol have been demonstrated to meet most of the stated criteria in algal growth inhibition tests. *t*-Butanol however is the less biodegradable one. Dimethylsulfoxide (DMSO) is a very efficient solvent, but might in some cases interact more easily with test substances and the test organism. Tests have shown that none of the solvents alone has any effect on algal growth up to a concentration of at least 1 ml/l (Reference [3]). In exceptional cases, higher solvent concentrations can be used to add higher concentrations of the test substance than possible with 100 µl/l.

It is recommended that a concentration series is prepared in the selected solvent, and that aliquots of the stock solutions are added to the test flasks, which already contain the algae and the test medium. Controls with and without the solvent shall be added to the test concentration series. The solvent concentration should be the same for all test solutions.

The solvent control group is the appropriate control group for comparisons with treated groups. Each group shall have the same solvent concentration as the control. For a bioassay in which a solvent is used in conjunction with the test chemical, the assumptions are that the solvent has no effect on the responses of

1) Ultra Turrax is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Schleicher & Schüll 604 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

interest and there is no interaction between the test chemical and the solvent. With the addition of a negative control (i.e. without solvent, as is required in all experiments using a solvent), the assumption regarding a solvent effect can be tested. However, unless the chemical is also tested in absence of a solvent, the assumption of no interaction between the solvent and the test chemical cannot be evaluated. Further guidance on the analysis of data from tests including solvent control can be found in Reference [18].

4.4 Dispersion using an emulsifying agent

The use of an emulsifier to prepare stock or test dispersions is generally the least preferable method. The nominal test concentrations may easily be considerably higher than the solubility limit in water, and the emulsifying agent may also influence the availability of a substance to the algal cells. However, if the exposure conditions with an emulsifier reflect the actual environmental exposure (e.g. pesticide formulations), and other addition methods appear to be impracticable, this method may be used. No dispersant should be added to formulated products.

Any emulsifier may be used if it meets the following requirements:

a) no inhibiting effects (direct or indirect) on algal growth at a concentration of 100 mg/l;

b) no or only slight biodegradation within a three-day exposure period;

c) no interference with the nutrient balance of the test medium.

The following emulsifying agents have been demonstrated to meet the stated criteria, but others may be used if required by the properties of the test substance:

— polyoxyethylene ethers [3];

— alkyl polyoxyethylene sorbitan [4];

— alkyl sorbitan [5].

A dispersion may be prepared by mixing appropriate amounts of the test substance and the chosen emulsifier by one of the methods described in 4.2. The concentration of the emulsifier should not exceed 100 mg/l. The selection of the best emulsifier is made by visually assessing the homogeneity of the stock dispersion.

Additional controls shall be added containing the same emulsifier concentration as in the test media. The use of the emulsifier controls in the data analysis is as described for solvent controls in 4.3.

4.5 Interference with algal growth and its measurement

If nominal test substance concentrations above the solubility limit or dispersions are tested, relatively high particle densities may occur in the test medium. High background particle numbers may disturb the growth measurements when using a particle counter or a spectrophotometer. For this reason, a background test substance concentration series without algae shall be included as a background correction of the measurements.

Usually quite high particle densities (i.e. at the same density level as the inoculum) are acceptable at the start of the test, as their influence on the subsequent measurements is progressively less due to the algal growth.

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3) Brij 56 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

4) Tween 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

5) Span 20 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

If treatments to reduce the particle densities (i.e. by filtration or centrifugation) should lead to a considerable loss of soluble substance, testing with particles present is preferred. In extreme cases, the growth can be determined by other methods or validated by counting of algal cells with a microscope.

Fluorimetric measurement of solvent extracted pigments (Reference [5]) may be an attractive indirect method of estimating the algal biomass, which eliminates interferences from particles. The method is however indirect as pigment content may vary with growth conditions.

Bacterial growth on biodegradable test substances or auxiliary substances (i.e. solvents or emulsifiers) cannot be prevented, as the algal cultures nearly always contain bacteria. The growth can be delayed however by working under aseptic conditions as much as possible and using sterilised equipment and media. A significant interference is expected only at the highest concentrations tested (i.e. in the range of 10 mg/l to 100 mg/l) of highly degradable substances [e.g. with a BOD_5/COD ratio [6)] of 0,5 or higher].

Solvents and in particular emulsifiers may inhibit or stimulate the algal growth. A stimulating effect is probably due to carbon dioxide or other nutrients released by degradation (at high cell densities the growth of the algal culture is often carbon limited). Stimulating effects may complicate the calculation of the EC values (see Clause 12).

5 Poorly soluble mixtures of organic substances

5.1 General

Mixtures of organic substances refer to both homogenous aggregates of a number of compounds with different physico-chemical and/or chemical properties, which cannot be easily separated into their component parts by physical means (e.g. oil products, mixtures of isomers), and formulated products (preparations such as formulated pesticides and oil based drilling fluids [3]).

The method of choice for testing mixtures containing poorly soluble substances and/or volatile substances is the preparation of Water-accommodated fractions (WAFs) by stirring and phase separation [2]. A WAF is an aqueous medium containing only that fraction of a substance which remains in the aqueous phase after the preparation procedure is terminated. Components of the test substance may be present either in true solution or as a stable emulsion. When filtered through suitable filters, Water-soluble fractions (WSFs) are obtained.

As the (assumed) equilibrium between the test substance and the aqueous phase depends on the test substance to liquid ratio, a WAF is prepared for each test concentration separately and should not be diluted. If however, stable dispersions are formed by the WAF preparation, these can further be treated according to 4.4.

In 5.2, the general preparation procedure for a WAF is described.

In testing WAFs, the results are expressed in terms of loading rates instead of the usual concentration term. Loading rate is the amount of test substance from which a WAF is prepared and is equivalent to the nominal concentration. The final results also shall be expressed as EL_{50}, and EL_{10} values, where L represents the loading rate.

5.2 Preparation of test media

Water-accommodated fractions (WAFs) are prepared by mixing the test substance with the algal growth medium at a range of loading rates in clean mixing vessels, using a suitable mixing apparatus. The mixing vessels shall be cylindrical and fitted with a drain port near the bottom for drawing off the WAF (commercially available aspirator bottles are quite acceptable). The mixing vessel volume shall be large enough to prepare the volume of WAF required for the exposure (and for sampling for analysis if relevant).

6) BOD = biochemical oxygen demand; COD = chemical oxygen demand.

The vessel volume has also to be small enough to minimize headspace whilst maintaining optimum surface contact between test material and the growth medium. The containers should preferably be sealed with ground glass stoppers, although PTFE-lined screw caps or tightly fitted, aluminium foil-covered neoprene stoppers may be acceptable. The loss of volatiles is prevented by tightly sealing the vessels, which should be incubated in the dark to prevent photochemical degradation of dissolved components.

A magnetic stirring bar (or other stirring apparatus) is placed in each vessel and the appropriate volume of algal growth medium added. The test substance is the last added to the surface of the medium being careful not to contaminate the sampling port. Mixing is initiated with the vortex in the centre extending approximately 1/3 from the top to bottom of the vessel. Care shall be taken not to draw a vortex of test material all the way to the bottom. If the test material appears to be forming an emulsion, the stirring speed should be reduced. Observations of the vortex depth and mixture appearance need to be made.

For test substances with a specific weight higher than the test medium, gentle, continuous shaking of the vessels should be applied during preparation of the WAFs.

The mixing period may be determined by carrying out an equilibration study (with analytical monitoring) under the conditions used to prepare the WAFs. As a guide, a mixing period of 20 h to 24 h has been found to yield a WAF containing dissolved components of hydrocarbons at equilibrium concentrations between aqueous and non-dissolved phases.

Following mixing, the contents of the vessels are allowed to stand undisturbed for 1 h to 4 h to allow separation of the aqueous and non-dissolved phases. The aqueous phase (the WAF) is then transferred directly into the test flasks.

Take care to ensure that any non-dissolved material is not transferred to the test vessels. The WAFs shall be tested as soon as possible, unless evidence is provided to demonstrate that their composition does not change during storage.

5.3 Test performance

Tests are started by the addition of a small volume of algal suspension to the WAFs. The WAFs usually contain a relative high number of particles that may prevent the use of a particle counter or a spectrophotometer for the determination of algal growth at high loading rates (test substance loading rates $\geqslant$ 10 g/l are required by some regulations).

Algal cell counting with a microscope of fluorometric measurements (see 4.5) can be used to determine the algal growth in such cases. However, if appropriate, WSFs can be tested instead of WAFs.

If the test substance contains appreciable amounts of biodegradable components, bacterial growth may be considerable. Its occurrence should be checked by microscopy (or by monitoring the appropriate channels or a particle counter) and, if relevant, a statement on its influence on the test results should be included in the test report (see also 4.5).

6 Poorly soluble inorganic materials

The materials considered in this clause may be solid metals, metal compounds, minerals, mineral containing wastes and mineral products. For such materials, WSFs should be prepared as described in 5.1 and 5.2. A guidance document for transformation/dissolution of metals and metal compounds is available from OECD [11].

For the WSF preparation, the materials shall be in a sufficiently fine powder to be dispersed in the test medium. Each test substance concentration is prepared separately by stirring until the equilibrium of relevant components in the test medium is reached. As guidance, a contact period of 20 h (in the dark at temperature range of the algal growth inhibition test) can be maintained. Thereafter, the suspensions are filtered through a membrane filter or centrifuged in order to remove particles that may disturb the measurement of cell density, and to prevent further leaching of components.

Each component of these materials has equilibrium solubility in the test medium, which depends on the composition of the algal growth medium and the solid/liquid ratio. At the usual concentrations tested (e.g. less than 1 000 mg/l), soluble metal compounds are usually the cause of algal growth inhibition (if any). The equilibrium concentration may be assessed either by analytical monitoring of the relevant element or by screening of algal growth. Metal compounds may have a complex interaction with the algal growth medium, which is further addressed in Clause 10.

Addition of one or more of the essential elements Co, Cu, Fe and Zn (already present in the test medium) to the test medium may stimulate algal growth under certain conditions.

The results of tests shall be expressed in terms of loading rates (see Clause 5), except when reference to measured soluble concentrations of a specific main component is required (e.g. the soluble metal concentration released by a low solubility metal oxide). In practice, the lowest loading rate that can be tested according to the method described is approximately 1 mg/l, and is limited by the particle size of the test material. Lower loading rates shall be obtained by dilution of the WSF of the lowest loading rate.

The leaching behaviour of components of mineral-containing solids may be very complex and is influenced by the solid to liquid ratio in the WSF preparation and the composition of the test medium. There are standard methods available for detailed characterization of leaching behaviour of solids that can provide guidance for the appropriate method to prepare a WSF with such materials (e.g. References [16] and [17]).

7 Volatile substances

7.1 General

A chemical substance may be characterized as volatile from aqueous solution if its Henry's law constant, H, is greater than about 1 Pa·m^3/mol and highly volatile if H is greater than about 100 Pa·m^3/mol. In order to obtain reasonably constant test substance concentrations in an algal growth inhibition test with highly volatile substances, closed test systems are needed. The testing of volatile substances can be carried out with partially filled closed bottles, whereas the testing of highly volatile substances requires testing in filled closed bottles [6].

A closed vessel that is partially filled limits, but does not eliminate, volatile loss from the aqueous phase. Substances of particularly high volatility may partition predominantly to the headspace, and a headspace-free test design should thus be used for highly volatile substance [6].

Optimum algal growth and the maintenance of a stable pH of the test medium both require a sufficient supply of CO_2. Atmospheric CO_2 can maintain CO_2 concentrations in an open test, whereas this is not possible in closed tests. There are a number of approaches to achieve a sufficient supply of CO_2 in closed test systems. The headspace can easily be enriched with 1 % to 2 % CO_2 using a syringe [7]. Alternatively, the dissolved CO_2 concentration in the medium can be increased by addition of, e.g. 0,3 g $NaHCO_3$/l and subsequent pH adjustment to pH 7,0 (Reference [6]). It is often desirable to reduce the initial algal density in such closed test systems, in order to allow a sufficient exponential growth period at a limited CO_2 supply.

It is required to base the calculation of EC values on measured concentrations.

7.2 Test system and growth medium

Bottles or vials with a volume of 20 ml to 300 ml which can be closed with a septum stopper are appropriate test systems. A septum allows samples to be taken with a syringe for chemical analysis or measurements of the cell density.

7.3 Test performance

The test performance depends on the material to be tested.

— Substances added by direct addition of liquids or as stock solutions in a solvent (see Clause 4)

  — The inoculated test medium is added to the test flasks, and

  — before or after their closure (depending on the volatility of the test substance), the appropriate volumes of test substance are added by pipetting or by syringe.

— Water-accommodated fractions (see Clause 5)

  — The water-accommodated fractions are carefully transferred to test bottles, which are then immediately closed to prevent losses of test substance as far as possible.

  — The bottles are opened only for inoculation with concentrated algal suspension.

— Gases

  — The gases are bubbled through sterile test medium for a sufficient period via a diffuser to obtain a saturated solution.

  — This stock solution can be diluted with test medium to obtain test solutions.

7.4 Interference with algal growth

The variations in the test conditions, notably the pH value, can be limited by several measures that are compatible with the validity criteria of ISO 8692 and ISO 10253.

The following measures may be taken:

a) decrease the light intensity to the lower level of the permitted range;

b) incubate at a lower temperature;

c) shake the test bottles continuously;

d) decrease the inoculum cell density.

The given measures aim at a lower growth rate and/or a more favourable biomass to carbon source ratio. The control growth rate should not however be lower than the validity criteria of ISO 8692 and ISO 10253.

The recommended algal species differ in cell size, but the inoculum is defined by cell density. This may lead to a relatively greater biomass-dependent pH increase in tests with the species *Desmodesmus subspicatus* (ISO 8692) and *Skeletonema costatum* (ISO 10253) than with the other recommended species.

The main effect of a carbon source limitation in a closed test system may be a lower control culture cell density at the end of the test and a shorter period of exponential growth compared with an open system. If the latter situation occurs, then the 72 h data can be ignored when assessing the effect on the growth rate.

8 Waste waters and environmental aqueous samples

The testing of waste waters is described in ISO 5667-16 and in Annex A of ISO 8692. In this International Standard, only aspects related to algal growth inhibition tests are mentioned.

Waste waters and environmental samples nearly always contain particulate matter and may be tested either before or after filtration.

Preferably, a complete sample is tested after time has been allowed for settling of coarse particulate matter. If further removal of particulate matter is necessary, centrifugation is preferred to filtration. The interference of particles on algal growth measurements and the effects of particles on algal growth are described in 4.5 and 5.3.

The decision to remove particles is influenced by the ability to measure algal growth under the prevailing conditions and whether the method for removing the particles changes the toxic effects or not.

The growth of bacteria may be substantial in wastewaters rich in organic substrate. Bacteria may interfere with the development of algal growth as well as with the measurement of cell density. In some cases, sterile filtration of the test sample may be necessary, although there is a risk of affecting the toxicity of the sample.

The initial pH of the test dilutions is not allowed to deviate more than 0,5 pH unit from the pH of the test medium specified by the relevant standards. If necessary, adjustment of the pH value may be carried out as described in ISO 8692 and ISO 10253. If the effect of the pH of the waste water is to be tested, the effect of pH adjusted and unadjusted samples may be compared in the test.

Add the same amounts of nutrient stock solutions to the undiluted sample as to the control growth medium before further dilutions with growth medium are prepared. In this way, all test batches receive the same concentrations of added nutrients, which allow optimal growth during the test and exclude effects of the nutrient content of the sample on the growth response. If a sample containing low salt concentrations is tested with a marine alga, algal growth may be negatively influenced by changes in the osmotic pressure of the test medium. In such a case, the relevant salt concentration (e.g. NaCl, artificial sea salts) is added to the undiluted sample before dilution with the growth medium.

If a salt-containing sample is tested with a fresh water alga, the influence of the salt may be assessed by an additional control series with appropriate salt concentrations.

Frequently, a stimulation of algal growth is observed with samples containing an increased concentration of nutrients and essential elements (compared with the test medium).

If a sample contains high concentrations of metal-complexing agents, the algal growth may be limited due to complexing of essential elements in the test medium, e.g. copper, cobalt, zinc and iron (see Clause 10).

The interferences of biodegradable substances, organic matter and algae in samples, and the connected bacterial growth are described in 4.5.

Analytical determination of the relevant components (see Clause 3) may help in the decision to amend the test sample with nutrients and other components.

9 Coloured and/or turbid samples

Light is an essential energy source for algal growth, and variation in light intensity may therefore influence the growth rate if the light intensity is the growth limiting factor. The change in growth rate caused by variation in the light intensity depends on whether the light intensity with which an algal culture is incubated is at the saturation intensity level or not. Above the saturation level, a change in light intensity does not change the growth rate. Below the saturation value, there is approximately a linear relationship between the light intensity and the growth rate (if no other factor is limiting the growth). The saturation light intensity is different for each of the algal species recommended by International Standards, and is not exactly known. It is assumed, however, that the lower end of the recommended light intensity range is below the saturation value.

Coloured and turbid (aqueous) samples and coloured substances and materials may therefore influence the algal growth negatively by shading or by filtering out a specific wavelength required by the algal cultures without having direct toxic effects in the same concentration range as the shading occurs. With a continuous shaken test system designed according to International Standards, practical experience has demonstrated

that significant shading effects are mainly observed with nearly opaque coloured solutions or turbid suspensions (continuous shaking assures that all algal cells are exposed to the full light intensity for a part of the testing period).

In order to further reduce the possible influence of shading on the growth rate of the test algae when very opaque or turbid samples are tested, the light intensity in the incubator may be increased to a level that assures a saturation light intensity at the highest concentration of the coloured and /or turbid test medium, in combination with a reduction of the thickness of the test medium layer [4], [12], [13], [14].

Correction for the shading effect by inserting filters between the light source and the cultures may lead to erroneous results and is not recommended [15].

10 Metals and metal compounds

10.1 Introduction

As with other bioassays, almost any result can be obtained from an algal toxicity test of heavy metals because the speciation of metals and hence their toxicity are affected by a number of physical-chemical factors that change with the test conditions. Generally the speciation of metals determine their bioavailability which again is a key determinant of the expressed toxicity. In addition to being affected directly by metal speciation, toxicity in itself may be influenced by chemical or physical-chemical factors such as the ratio between Ca^{2+} and Mg^{2+}, and most notably by pH, because hydrogen ions compete with heavy metals for sorptive sites on the algal surface (this pH effect on toxicity is additional to the parallel toxicity modifying effect of pH that is due to the influence on metals speciation).

Algal tests pose a number of special problems not encountered in for instance toxicity tests with aquatic animals. A particular problem inherent in algal tests is that addition of iron, trace metals and chelator to the test medium is necessary to ensure adequate growth of the algae under the test conditions. Iron and trace metals are nutritional needs and a chelator shall be added to keep iron in solution. These medium constituents unavoidably change heavy metal toxicity — not only due to the chelator but also as a result of interactions between the chelator and the other medium constituents, iron in particular. The growth of algae as such brings about further changes, which influence heavy metal toxicity. Most important here are pH-drift and release of algal exudates which complex or in other ways associate with metals. Finally, metals are sorbed onto the algal cells, causing a decrease in toxicity at high biomass levels by reducing the toxic exposure per unit of biomass or per cell.

The algal test system itself thus influences heavy metal toxicity, and changes in toxicity inevitably occur during the course of the test. However, with proper optimization, algal tests on heavy metals can in fact be carried out under conditions where the influence by the test system is minimal, so that reproducible results can be obtained that unambiguously refer to the test material and the physical-chemical characteristics of the test medium. Key factors in such optimisation are that the algal biomass is kept low, that the influence of the chelator, iron and trace metals is minimized, and that pH is well controlled. A suggested modified test procedure for the freshwater algae growth inhibition test (ISO 8692) is described in 10.2.1 to 10.2.4.

10.2 Modification of algal growth inhibition test procedures for testing materials containing heavy metals

10.2.1 Growth medium

The growth medium should be modified to minimize the reduction in metal toxicity due to chelator. In the fresh water test (ISO 8692), this can be achieved firstly by minimizing the chelator concentration and secondly by using stoichiometrically-balanced amounts of chelator and iron plus trace metals Zn, Co and Cu, respectively.

The critical factor in this optimization is to have an iron concentration large enough to ensure maximum growth of algae during the entire test. Bioassays with *Pseudokirchneriella subcapitata* have revealed that starting with 10^4 cells/ml, an iron concentration of 20 µg/l (0,074 µM) of $FeCl_3 \cdot 6H_2O$ is well in excess of the demands for a two day test period even with high growth rates. Further, the trace metal spike should be reduced to one third

of the level of ISO 8692 which corresponds to a combined concentration of Co, Cu and Zn of 0,009 46 µM. Co, Cu and Zn are those trace metals that compete with the heavy metals of the test material for chelator sites. To minimize the complexation of the test material, the chelator is added in a concentration exactly equivalent to that of iron and the three "trace heavy metals", or 0,083 4 µM with equimolar binding, leaving no excess chelator capacity. Therefore, the test material metals shall displace Fe, Co, Cu or Zn from their complexes before they can associate with chelator ligands.

10.2.2 Chelator

With EDTA as the chelator, the equimolar amount to the trace metal spike specified in 10.2.1 is 38,1 µg/l of $Na_2EDTA{\cdot}2H_2O$, which is a reduction of the amount used in ISO 8692 by a factor 2,6. EDTA is used in accordance with current practice in algal toxicity testing and culturing, but EDTA is not ideal for use with toxicity testing of metals. Firstly, it has been found that the coloured complex FE(III)EDTA photodecomposes partly under the conditions of an algal test, thus changing the metals speciation (iron is probably kept bioavailable by algal exudates). EDTA also forms very strong complexes, not only with iron, but also with other metals and the stability constants exceed by far those of complexes with natural chelating agents in water.

Citric acid is an alternative chelator which forms weaker complexes with metals and which is used in algal growth media. The equimolar concentration of citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$) is 23,1 µg/l.

10.2.3 Test period

In order to restrict the final algal biomass density, the test period in tests of metal toxicity should be reduced to two days. With the fast-growing standard green algal species, *Pseudokirchneriella* and *Desmodesmus* and the standard inoculum 10^4 cells/ml, the final biomass would otherwise be too high for testing the toxicity of metals. An alternative solution is to reduce the inoculum density, for example to 10^3 cells/ml.

10.2.4 Control of pH

Strict control of pH is extremely important in tests of metals, because pH influences both metal speciation and metal toxicity as such. Maintaining a constant pH in algal cultures often causes difficulties, but the reduction of test period or inoculum density as described in 10.2.3 has the effect of reducing pH shifts in the test medium, because the biomass density is restricted. Refer also to Clause 11.

11 pH buffering

The pH value of the test medium can influence toxicity considerably. A pH-change of one unit, for example, can easily give rise to a change in toxic concentration levels by an order of magnitude for metals and ionizable organic compounds, and pH shall therefore generally be controlled in algal toxicity tests if the results are to be reproducible and relate to a specific pH.

Control of pH is a general difficulty of algal toxicity testing due to the fact that the CO_2 used for photosynthesis is largely supplied from an external gas phase and not directly present as a medium constituent. The mass transport of CO_2 across the water/gas interface is rather slow however, and with a sufficient utilization rate of CO_2 by the algae, the dissolved CO_2 concentration drops below the concentration in equilibrium with the gas phase. This leads to a displacement of the carbonate system towards dissolved CO_2 and carbonic acid, by which process, OH-ions are formed leading to a temporary pH-increase. Permanent pH changes are minor and are caused by uptake of nitrogen species (NH_4^+, NO_3^-) and phosphate ions, liberating H^+ and OH^- ions. Thus control of pH in algal cultures is first of all a matter of ensuring a gas phase/water CO_2 mass transport potential that exceeds the algal demand for CO_2.

With the medium prepared according to ISO 8692, the pH in fact slightly decreases with time if the mass transport conditions are good, because the utilization of ammonia as the nitrogen source leads to release of H^+ ions. Assuming a constant proportion of CO_2 in air, the equilibrium pH of the ISO 8692 medium with 50 mg/l of $NaHCO_3$ can be calculated to be pH 8,1. With only bicarbonate and air as a buffering system, pH-values in the range 7 to 9 can be obtained. With 15 mg/l $NaHCO_3$, for example, the equilibrium pH is 7,5. For lower pH values, the procedure described for volatile test materials (Clause 7), based on a closed flask system with a CO_2-enriched headspace, can be utilized.

Sometimes the addition of synthetic buffers can be helpful in controlling pH very strictly, in particular at pH values at/or below neutral. The so-called zwitterionic biological buffers contain both positive and negative ionisable groups. The positive charges are provided by secondary and tertiary amine groups, the negative by sulfonic and carboxylic acids. The buffers do not interfere with chemical and biochemical processes involved in the algal growth as phosphate buffers possibly do. Recommended buffers are MES [7] or HEPES [8]. They are recommended at a concentration of 12,5 mmol/l.

Pre-cultures shall be cultivated in the test medium with the same buffer concentration as used in the test.

Buffers are added to the medium before sterilization. There is experimental evidence that autoclaving does not influence the buffering capacity.

12 Interpretation of the results

The results of the growth inhibition tests can be treated by the methods given in the ISO 8692 and ISO 10253. The calculation of the LID-value [9] is described in ISO 5667-16. If the tests are carried out with WAFs, the term loading rate (i.e. EL_{50} and EL_{10} values) shall replace the concentration terms (see 5.1).

Some groups of substances for which this International Standard is relevant may not be stable during the test or may absorb strongly to the algal biomass. In such cases, the growth curves of cultures exposed to the test substance may deviate from the exponential pattern. A typical effect in such cases is an extended lag-phase before growth proceeds at the control growth rate. Such effects should be reported when observed.

Stimulation of growth compared with the control is often observed in algal growth inhibition tests, particularly in tests with wastewater. Because it is difficult in each case to assume any causal relationship between test substance and growth stimulation, the stimulation is not regarded as an effect. Cell densities higher than in the control are treated as if they are identical to the control.

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7) MES = 2-(*N*-morpholino)ethanesulfonic acid. pK_a = 6,15.

8) HEPES = 2-[(4-hydroxyethyl)-1-piperazine]ethanesulfonic acid, sodium salt. pK_a = 7,55.

9) LID = Lowest ineffective dilution.

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