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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

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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.