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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 matières peu solubles, composés volatiles, métaux et eaux résiduaires

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 14442 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annex A of this International Standard is for information only.

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

1 Scope

This International Standard provides guidelines for testing substances for algal growth-inhibition that are difficult to test and thus not covered by the methods described in ISO 8692 and ISO 10253.

Guidelines are given for preparing the substance for testing and for carrying out an appropriate test. This International Standard is applicable to the following test substances:

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 analytical procedures and interpretation of results have also been included.

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

2 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 to regulatory authorities. Such activities are usually not an integral part of International Standards concerning algal growth inhibition test methods.

However, there may be situations where analysis may assist in defining the appropriate exposure conditions for 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 (log*Pow*), Henry's constant, photochemical and hydrolytic stability, and biodegradability.

Analytical confirmation is strongly recommended in order to verify test substance concentrations, and is required for the calculation of EC values of volatile substances (see clause 6). If losses due to adsorption on the test vessels or during transfer of test solutions and media occur, then analytical confirmation is of particular importance. This aspect is also addressed in ISO 5667-16.

Due to the static 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) sterilize media and equipment to reduce the effect of bacterial growth;

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

c) avoid contact with water prior to testing to reduce hydrolytic decomposition;

d) treat glassware (e.g. silanization);

NOTE The effectiveness of such a treatment varies from chemical to chemical.

e) pre-condition the glassware with solutions at the test concentrations before addition of the test media.

The effects of such technical measures are, if relevant or possible, monitored by chemical analysis.

Water, waste water and organic/inorganic solids/liquids may contain components that can modify the composition of the algal growth medium (e.g. by precipitation of a limiting nutrient, complexation of essential elements, addition of nutrients) and subsequently cause effects on algal growth not related to any 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 test substance contains a high concentration of readily degradable organic material, subsequent bacterial growth can disturb the algal growth measurement. When untreated (not filtered or centrifuged) waste water is tested, contamination with other algal species can occur.

3 Poorly soluble pure organic substances

3.1 General

A pure substance is a substance containing one major component, with minor components as impurities. A poorly soluble substance is one with a solubility limit below 100 mg/l in water. If, however, growth inhibition occurs at concentrations much lower than the solubility in water, 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 of a very low solubility).

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

Testing of concentrations nominally 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 [3] (see Bibliography):
Water solubility below 100 mg/l: "sparingly soluble".
Water solubility below 1 mg/l: "very low solubility".

A number of methods 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.

3.2 Preparation of saturated 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 to the test medium. A saturated solution is usually prepared by stirring (e.g. using a magnetic stirrer or shaking; see also 4.1) an excess amount of the test substance in test medium for a period of time. 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 in which 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 membrane filter of pore size 0,45 μm) or centrifugation may be useful for removing particulate matter.

NOTE Certain membrane filters may interfere with the test substance. The filter should be chosen according to the physicochemical 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.

NOTE 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 to ensure 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 should 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[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.

3.3 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 will not influence the solubility of substances but assist 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 3.2 to prepare saturated solutions.

1) "Ultra Turrax" is an example of a suitable product available commercially. This information is given for the convenience of users of ISO 14442 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 ISO 14442 and does not constitute an endorsement by ISO of this product.

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

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 is obtained by 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. 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 (see ref. [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 be prepared in the selected solvent, and that aliquots of the stock solutions be added to the algae-containing test medium already present in the test flasks. Controls with and without solvent shall be added to the test concentration series. The solvent concentration should be the same for all test solutions.

3.4 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:

a) polyoxyethylene ethers[3];

b) alkyl polyoxyethylene sorbitan[4];

c) alkyl sorbitan[5].

3) Brij 56 is an example of a suitable product available commercially. This information is given for the convenience of users of ISO 14442 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 ISO 14442 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 ISO 14442 and does not constitute an endorsement by ISO of this product.

A dispersion may be prepared by mixing appropriate amounts of the test substance and the chosen emulsifier by one of the methods described in 3.2. 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.

3.5 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 should 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 algal growth.

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

Fluorimetric measurement of solvent-extracted pigments (see ref. [5]) may be an attractive indirect method of estimating the algal biomass, which eliminates interferences from particulates. The method however is 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 will nearly always contain bacteria. The growth can be delayed, however, by working under aseptic conditions as much as possible and by using sterilized equipment and media. 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 five-day biological oxygen demand (BOD_5)/COD ratio of 0,5 or higher).

Solvents and emulsifiers in particular may inhibit or stimulate 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 10).

4 Poorly soluble mixtures of organic substances

4.1 General

Mixtures of organic substances refer to both homogeneous aggregates of a number of compounds with different physicochemical and/or chemical properties, which can be separated by physical means (e.g. oil products, mixtures of isomers), and to formulated products (such as formulated pesticides and oil-based drilling fluids) (see ref. [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 (see ref [2]). A WAF is an aqueous medium containing only that fraction of a substance which remains in the aqueous phase after the preparation procedure has been 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 ratio of test substance to liquid, 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 in accordance with 3.4.

In 4.2 the general procedure for preparation of a WAF is described.

In testing WAFs, the results are expressed in terms of the loading rate instead of the usual concentration units. The loading rate is the amount of test substance from which a WAF is prepared, and is equivalent to the nominal concentration. The final results shall also be expressed as EL_{50}, EL_{10} and NOEL values, where EL represents the loading rate and NOEL represents the No Observed Effect Loading rate.

4.2 Preparation of test media

Prepare water-accommodated fractions (WAFs) 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 should be cylindrical and fitted with a drain port near the bottom for drawing off the WAF (commercially available aspirator bottles are quite acceptable). The volume of the mixing vessel should be large enough to contain the volume of WAF required for the exposure (and for sampling for analysis if relevant). The vessel volume shall also be small enough to minimize headspace whilst maintaining optimum surface contact between test material and growth medium. The container 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 vessel should be tightly sealed to prevent loss of volatiles and should be incubated in the dark to prevent photochemical degradation of dissolved components.

Place a magnetic stirring bar (or other stirring apparatus) in each vessel and add the appropriate volume of algal growth medium. Add the test material last to the surface of the medium, being careful not to contaminate the sampling port. Initiate mixing with the vortex in the centre extending approximately one third of the distance from the top to bottom of the vessel. Take care 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. Make observations of the vortex depth and mixture appearance.

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 undissolved phases.

Following mixing, allow the contents of the vessels to stand undisturbed for 1 h to 4 h to allow separation of the aqueous and undissolved phases. Then transfer the aqueous phase (the WAF) directly into the test flasks.

Take care to ensure that any undissolved material is not transferred to the test vessels. Test the WAFs as soon as possible unless evidence is provided to demonstrate that their composition does not change during storage.

4.3 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 required by some regulations).

Algal cell counting with a microscope 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 of a particle counter) and, if relevant, a statement on its influence on the test results should be included in the test report (see also 3.5).

5 Poorly soluble solid 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 4.2.

For the WSF preparation, the materials should be in a powder sufficiently fine to be dispersed in the test medium. Prepare each test substance concentration separately by stirring until the equilibrium of relevant components in the

test medium has been reached. As a guide, a contact period of 20 h (in the dark at the temperature range of the algal growth inhibition test) can be maintained. Thereafter, filter the suspensions 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.

The equilibrium solubility of each component of these materials in the test medium will depend on the composition of the algal growth medium and the solid/liquid ratio. At the usual concentrations tested (e.g. less than 1000 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 9.

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.

Express the results of tests in terms of loading rates (see clause 4), except when reference to measured soluble concentrations of a specific main component is required (e.g. the soluble metal concentration released by a metal oxide of low solubility). In practice, the lowest loading rate that can be tested in accordance with the method described is approximately 1 mg/l, and is limited by the particle size of the test material. Lower loading rates can be obtained by dilution of the WSF of the lowest loading rate.

NOTE The leaching behaviour of components of minerals 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. Standard methods are 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. refs. [9] or [10]).

6 Volatile substances

6.1 General

A chemical substance is characterized as volatile from aqueous solution if the Henry's law constant, H, is greater than about 1 Pa·m^3/mol, and as 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 volatile substances, closed test systems are needed. Optimum algal growth in the test system depends on the exchange of CO_2 with air. The growth conditions, in particular the pH value of the test medium, may deviate strongly from those defined for open systems by the relevant International Standard. This problem is obviously most important with the less-buffered freshwater medium used in application of ISO 8692.

Carry out testing of volatile substances with partially filled closed bottles and a buffered algal growth medium to maintain the pH conditions.

The purpose of the buffering is to keep the normal pH increase in the control cultures within the limits prescribed in the relevant standards, and extend the exponential growth phase sufficiently to calculate the effects on the growth rate. The calculation of EC values shall be based on measured concentrations.

NOTE A closed vessel that is partially filled will limit, but not eliminate, volatile loss from the aqueous phase. Substances of particularly high volatility may partition predominantly to the headspace using the system described above.

6.2 Test system and growth medium

Bottles (of maximum volume 250 ml) filled with 100 ml of test solution and closed by a stopper with a septum are appropriate test systems. Although the test system is closed, sampling of the test solution is still possible by using a syringe. In this way chemical analysis or measurements of the cell density can be conducted during the study. A sampling method is presented in annex A.

In standard algal growth inhibition tests in open flasks, carbon dioxide from the air is available as a carbon source. In closed systems an additional carbon source should be available to obtain an appropriate growth of the algae. By using a concentration of 0,3 g $NaHCO_3$ per litre medium, a sufficient increase in cell density is expected.

If an extreme increase in pH occurs, addition of 6 mM HEPES[6)] buffer has been shown to buffer the pH value sufficiently. The non-physiological HEPES buffer is chosen instead of a phosphate buffer because increased concentrations of the latter buffer will influence algal growth.

NOTE An alternative would be to enrich the head space with 2 % to 3 % carbon dioxide.

6.3 Test procedure

The test procedure depends on the material to be tested, i.e.:

— substances added by direct addition of liquids or as stock solutions in a solvent (see clause 3);

Add the inoculated test medium to the test flasks and before or after their closure (depending on the volatility of the test substance and the buffer system selected) and add the appropriate volumes of test substance by pipetting or by syringe.

— water-accommodated fractions (see clause 4);

Carefully transfer the water-accommodated fractions to test bottles, then immediately close them to prevent losses of test substance as far as possible. Open the bottles only for inoculation with concentrated algal suspension.

— gases.

Bubble gases 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.

6.4 Interference with algal growth

Variations in test conditions, notably the pH value, can be controlled 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 slightly.

The measures given aim at a lower growth rate and/or a more favourable biomass-to-carbon-source ratio. Data from interlaboratory ring tests demonstrated that the endpoint of the growth inhibition test was insensitive to variations of the control growth rate. 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 *Scenedesmus 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 growth rate. Due to the growth pattern of the marine algae *S. costatum,* ISO 10253 allows the deletion of the 72 h data with this species.

6) HEPES = 2-[(4-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, sodium salt, pKa = 7,55.

7 Waste waters and environmental samples containing water and sediments

The testing of waste waters is described in ISO 5667-16. In the present International Standard, aspects related only 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 is described in 3.5 and 4.3.

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 will change the toxic effects or not.

The initial pH of the test dilutions shall not deviate more than 0,5 pH units from the test medium pH 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 pH of the waste water is to be tested, the effect of pH-adjusted and -unadjusted samples may be compared in the test.

If undiluted samples or concentrations higher than 10 ml/l are tested and the water sample lacks essential nutrients for algal growth, algal growth may be lower than in the controls due to a lack of nutrients in the test dilutions. For this purpose, nutrient stock solution is added to the undiluted sample and control samples to achieve the same final nutrient concentrations before further dilutions with test medium are prepared. 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 sample containing salt is tested with a fresh water alga, the influence of the salt can be assessed by an additional control series with appropriate salt concentrations.

Frequently, 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, algal growth may be limited due to complexing of essential elements in the test medium, e.g. copper, cobalt, zinc and iron (see clause 9).

The interferences of biodegradable substances, organic matter and algae in samples, and the related bacterial growth, is described in 3.5.

Analytical determination of the relevant components (see clause 2) may aid in the decision to amend the test sample with nutrients and other components.

8 Coloured and/or turbid samples

Light is an essential source of energy for algal growth, and variation in light intensity may therefore influence the growth rate if light intensity is the growth-limiting factor. The growth rate of an algal culture will increase with light intensity up to a light saturation value. Above the saturation level, a change in light intensity will not change the growth rate. Below the saturation value, there is an approximately linear relationship between the light intensity and the growth rate (if no other nutrient is limiting the growth). The saturation light intensity is different for each of the algal species recommended by the relevant International Standard, and is not exactly known. It is assumed, however, that the recommended light intensity range is below the saturation value.

Coloured and turbid (aqueous) samples and coloured substances and materials can 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 at which the shading occurs. With a continuously shaken test system designed in accordance with the relevant 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 test period).

In order to distinguish quantitatively between shading effects and inhibition effects, one of the following additional control tests may be carried out:

— increase the light intensity in the incubator to a level that assures saturation light intensity at the highest concentration of the coloured and/or turbid test medium, in combination with a reduction in thickness of the test medium layer (see ref. [4]);

— simulate the shading effect by using the test media without algae as a filter for algal cultures.

Either option may lead to unavoidable deviations in test conditions compared with the standard conditions.

In the first option, the saturation light intensity needs to be determined separately for each test species. The light intensity can exceed the range specified in the relevant International Standard, and may cause an increase in the pH value of the control batch. The control test is further carried out in accordance with the relevant International Standard.

In the second option (often more compatible with the relevant International Standards), the manipulations with the test design may lead to a lower light intensity, and therefore to a relatively lower control growth rate. However, interlaboratory ring tests have demonstrated that, as long as the control growth rate is above the validity limit given in the relevant International Standard, the EC_{50} values do not change significantly with changes in the control growth rate.

If a spectrophotometric method is used for cell density determination, a control series of the test substance without algae should be included.

One example of how a simulation may be designed is given below:

a) Algal cultures in standard test flasks are placed in glass beakers. The distance between the beaker wall and the test flask should be about half the diameter of the test flask.

b) The beaker is then filled to at least the level of the algal culture with test substance solutions/dispersions which then act as filters. A control with algal growth medium as a filter should also be included.

c) Each beaker is then covered with aluminium foil. The aluminium foil covers the top of the beaker and extends downwards to the level of the filter liquid in order to ensure that light passes only through the filter liquids before reaching the algal cultures.

d) The test flasks may be incubated without continuous shaking, e.g. with only stirring by hand at least twice a day.

9 Metals and metal compounds

9.1 General

The effect of metal compounds on algal growth has been shown to depend on the speciation and in particular on the concentration of its free ion in the algal growth medium. The free ion concentration depends on the extent of complexation with organic and inorganic ligands. Element speciation and concentration of free ions can be established with speciation models with full consideration of molar balances, relevant thermodynamic equilibrium constants, ionic strength, pH and concentration of the chelating agent introduced into the algal growth medium.

The interaction between metals, medium components and algal growth is complex. A number of metals, notably cobalt, copper, zinc and iron, are essential elements required for optimum growth of the algae. Changes in their free ion concentration may influence the algal growth rate if concentration of a specific element is the growth-limiting factor. Algal growth medium usually contains a low concentration of chelates in order to assure a sufficient availability of essential elements, and iron in particular, for the algal growth. Generally it is not known which element of the standard algal growth medium is the growth-limiting factor.

The addition of excess chelating agent may lower the free ion concentration sufficiently to cause a nutrient-limited decrease in the growth rate. Algal growth inhibition tests with chelating agents should therefore not be carried out with the free acid or a salt of the chelating agent, but with its metal complex (preferably an iron complex, as iron usually has the higher complex stability constant).

Furthermore in a situation where the ligand concentration is the limiting factor (as is the case with the standard algal growth medium), the free ion equilibrium concentration will depend on the relative complex stability constant of the metals and their concentration.

EXAMPLE If the copper concentration is increased in the standard test medium, the copper will replace zinc in the EDTA complexes, as copper has the higher stability constant of these elements. The consequence is a relatively high increase in the free zinc ion concentration compared with the free copper ion concentration.

All these factors make it difficult to design a test system that in all cases unequivocally demonstrates the effects of a specific metal on algal growth, and to extrapolate these effects to the surface water. In surface water, however, similar factors operate in complexing of metals. Metals are often present as organic complexes, resulting in a supply of metal ions originating from the equilibrium involved.

Therefore a test for metals has been designed in which the natural processes of speciation are simulated by adding synthetic complexing agents to the standard algal growth medium. Variation in the pH value of the test medium is also considered relevant to the determination of metal toxicity. Therefore the possibility is provided for buffering the test medium at the pH value which is relevant for the freshwater algae involved. By this method it is possible to determine the comparative effects of metals on algal growth.

The detailed test design and the interpretation and extrapolation of results should be based on speciation model calculations (analytical chemistry computer programs, see refs. [6] to [8]). There is experimental evidence that these models are able to predict the behaviour of metals in the test medium in relation to their growth-inhibiting effects.

9.2 pH buffering

The so-called zwitterionic biological buffers are useful for pH buffering of the algal growth medium. These buffers contain both positive and negative ionizable 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 can. Buffers MES[7)] or HEPES are recommended at a concentration of 12,5 mM.

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.

9.3 Metal buffering

The concentration of dissolved metals can be regulated by complexing agents such as ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA). For testing metals, NTA is preferred since its binding constant with calcium is 10^6 times lower than that with EDTA and its stability is higher under the prevailing test conditions. With NTA, a lack of biologically available Ca^{2+} at low heavy metal concentrations is avoided; Ca^{2+} deficiency may influence the cell wall permeability and the uptake of heavy metal ions.

NTA influences all dissolved ion concentrations, and addition of the metal under investigation may influence these concentrations. To prevent these changes, NTA shall be present in excess. The maximum concentration of NTA which has no significant effect on growth parameters is $1{,}8 \times 10^{-3}$ M. NTA can be added to the growth medium before sterilization.

7) MES = 2-(*N*-morpholino)ethane sulfonic acid, pKa 6,15.

10 Interpretation of results

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

The group 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 are considered to depend on the algal biomass present in the test system. Investigations indicate that the EC_{50} accurately describes the short-term and transient effects of substances on algal inoculum.

Stimulation of growth compared with the control is often observed in algal growth inhibition tests, particularly in tests with waste water. 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 the control are treated as being identical to the control