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Water quality — Sampling —

Part 16:
Guidance on biotesting of samples

Qualité de l'eau — Échantillonnage

Partie 16: Lignes directrices pour les essais biologiques des échantillons

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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.

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.

International Standard ISO 5667-16 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 6, *Sampling.*

ISO 5667 consists of the following parts, under the general title *Water quality — Sampling*:

— *Part 1: Guidance on the design of sampling programmes*

— *Part 2: Guidance on sampling techniques*

— *Part 3: Guidance on the preservation and handling of samples*

— *Part 4: Guidance on sampling from lakes, natural and man-made*

— *Part 5: Guidance on sampling of drinking water and water used for food and beverage processing*

— *Part 6: Guidance on sampling of rivers and streams*

— *Part 7: Guidance on sampling of water and steam in boiler plants*

— *Part 8: Guidance on the sampling of wet deposition*

— *Part 9: Guidance on sampling from marine waters*

— *Part 10: Guidance on sampling of waste waters*

— *Part 11: Guidance on sampling of groundwaters*

— *Part 12: Guidance on sampling of bottom sediments*

— *Part 13: Guidance on sampling of water, wastewater and related sludges*

— *Part 14: Guidance on quality assurance of environmental water sampling and handling*

— *Part 15: Guidance on preservation and handling of sludge and sediment samples*

— *Part 16: Guidance on biotesting of samples.*

Annexes A and B of this part of ISO 5667 are for information only.

Introduction

Biological tests are suitable for determining the effect of chemical and physical parameters on test organisms under specific experimental conditions. In principle, the methods of chemical analysis are not suitable for determining the biological effects. These effects can be enhancing or inhibiting, and can be determined by the reaction of the organisms, e.g. death, growth, proliferation, morphological, physiological and histological changes. Inhibiting effects are triggered by toxic water constituents or by other noxious influences.

Effects can refer to various levels, e.g. proceeding from (sub)cellular structures or enzyme systems, concerning the whole organism, and eventually the supra-organism or community level.

In the context of this part of ISO 5667, toxicity is the ability of a substance to exert a deleterious effect on organisms or biocenoses due to its chemical properties and its concentration.

The deleterious potential of a toxic substance can be counteracted by the protective potential of the biological system, for instance by metabolic detoxification and excretion. The apparent toxicity measurable in the biological test is the result of the interaction between the substance and the biological system.

Apart from the direct toxic effect of one or more water constituents, damaging biological effects can be exerted by the combined action of all noxious substances, e.g. by substances which are not toxic *per se* but affect the chemical or physical properties of the medium and, consequently, the living conditions for the organisms. This applies for instance to oxygen-depleting substances, coloured substances or turbid matter which reduce light exposure. It also includes non-substance-related effects such as impairment or damage due to extreme temperature.

Biological tests also include those tests which examine the effect of organisms on substances, e.g. microbial degradation studies.

The results of the biological tests refer primarily to the organisms used in the test and the conditions stipulated in the test procedure. A harmful effect stated by means of standardized tests can justify concern that aquatic organisms and biocenoses might be endangered. The results, however, do not permit direct or extrapolative conclusions as to the occurrence of similar effects in the aquatic environment. This applies in particular to sub-organism systems, as important properties and physiological functions of intact organisms (e.g. protective integuments, repair mechanisms) are removed or deactivated.

In principle there is no organism and no biocenosis which can be used to test all the effects on the ecosystem possible under the various

constellations of abiotic and biotic conditions. Only a few ("model") species representing relevant ecological functions can be tested in practice.

Besides these fundamental and practical limitations in the selection of test organisms, the sample to be tested can also pose experimental problems on biotesting. Waters, in particular waste waters, are complex mixtures and often contain sparingly soluble, volatile, unstable, coloured substances and/or suspended, sometimes colloidal, particles. The complexity and heterogeneity of materials give rise to a variety of experimental problems when performing biotests.

Special problems are related to the instability of the test material due to reactions and processes such as:

— physical (e.g. phase separation, sedimentation, volatilization);

— chemical (e.g. hydrolysis, photodegradation, precipitation); and/or

— biological (e.g. biodegradation, biotransformation, biological uptake in organisms).

Other problems, especially if spectrometric measurements are applied, relate to turbidity and colour.

This part of ISO 5667 is one of a group of International Standards dealing with the sampling of waters. It should be read in conjunction with the other parts and in particular with ISO 5667-1, ISO 5667-2 and ISO 5667-3.

Water quality — Sampling —

Part 16:
Guidance on biotesting of samples

1 Scope

This part of ISO 5667 gives practical guidance on sampling, pretreatment, performance and evaluation of waters in the context of biotesting. Information is given on how to cope with the problems for biotesting arising from the nature of the water sample and the suitability of the test design.

It is intended to convey practical experience concerning precautions to be taken by describing methods successfully proven to solve or to circumvent some of the experimental problems of biotesting of waters.

Reference has been made as far as possible to existing International Standards and guidelines. Information taken from published papers or oral communication is utilized as well.

Primarily dealt with are substance-related problems concerning sampling, pretreatment and preparation of water samples for biotesting and treatment of samples during the test, especially when performing tests with waters and waste waters containing unstable or removable ingredients. Basic principles of quality assurance, evaluation of data and presentation of results are outlined.

Special emphasis is laid on ecotoxicological testing with organisms ('single-species biotests'). Some features addressed in this general guidance apply as well to biodegradation and/or bioaccumulation studies as far as sampling and sample preparations is concerned. Preparation of poorly soluble substances and testing beyond the water-solubility limit is also addressed.

This part of ISO 5667 is not applicable to bacteriological examination of water. Appropriate methods are described in other International Standards.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 5667. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 5667 are encouraged to investigate the possibility applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 5667-3 :1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 5667-10 :1992, *Water quality — Sampling — Part 10: Guidance on sampling of waste waters.*

3 Sampling

3.1 General

The choice of representative sampling points, frequency of sampling, type of samples taken, etc. is dependent on the objective of the study. In general, the sampling approach for chemical analysis is compatible with the purpose of biotesting.

Some tests, however, require the water and waste water to be handled and kept in a particular way.

Depending on the type of investigation (e.g. toxicity or biodegradation tests) and the way the samples are to be processed, it is necessary to divide a sample into different portions which are preserved and/or stored under different conditions and processed in different ways.

If several samples have been taken (e.g. from different locations or at several times) they may be combined to achieve greater representativity. These samples should be thoroughly mixed and, if necessary, divided into subsamples. To obtain subsamples of equal quality, it should be ensured that the bulk sample maintains homogeneity during the subsampling process, e.g. by continuous shaking or stirring. This holds particularly in the case of two-phase mixtures, e.g. waters containing suspended particles, algal suspensions. It is recommended to use cooling sampling apparatus when several samples taken at several times are combined.

3.2 Samplers/vessels/containers

The volume, shape and material of the vessels are dependent on the nature of the sample (e.g. degradability/stability), the number of replicates, the volume required for these tests and the necessity of preserving and storing the samples prior to further processing.

The time required for freezing and thawing should be minimized by reducing the sample volume, i.e. the size of the vessel. In general it is appropriate to use one-litre vessels for freezing. For tests requiring larger volumes, the sample should be divided into vessels holding not more than 10 l.

The total sample volume taken should be sufficient to cover any supplementary or repeated testing. Remaining subsamples stored frozen separately should be saved until the final evaluation has been made.

The material of vessels should be chemically inert, easily cleaned and resistant to heating and freezing. Glassware, polyethene or polytetrafluoroethene (PTFE) vessels are recommended.

3.3 Filling status of containers

It should be decided whether the containers should be filled completely to the brim or only partially, having an air space, by taking into account the type of sample, the preservation mode and the biotest envisaged.

Problems related to partial filling can be

— enhanced agitation during transport, leading to breakdown of aggregated particles;

— interaction with gas phase, leading to stripping;

— oxidation of substances, leading e.g. to precipitation of compounds of heavy metals.

Problems related to complete filling can be

— oxygen depletion, with possible decomposition, leading to formation of toxic metabolites (e.g. nitrite, sulfide);

— impairment of homogenization by shaking or stirring the total volume.

Sample containers, when freezing is envisaged for preservation, should not be filled completely in order to allow expansion of volume.

4 Transport

The samples collected should be protected from breakage, temperature increase and external contamination. Misidentification of samples transported in melting ice should be avoided by using waterproof markers and/or labels.

5 Preservation and storage

As stated in ISO 5667-3, it is impossible to give absolute rules for preservation, e.g. the duration of possible storage and efficiency of various modes, because it depends primarily on the nature of the sample, especially its biological activity.

Potable waters and ground waters are generally less susceptible to biological and chemical reactions than surface waters, treated or raw waste waters. If the chemical composition can be approximately anticipated, reference should be made to ISO 5667-3 for the purposes of biotesting. Some additional precautions, however, should be considered as follows.

Samples for biotesting should be processed preferably without delay after collection to avoid changes in the original composition as a result of physical and chemical reactions and/or biological processes. The maximum duration of storage should not exceed 12 h at ambient temperature (maximum 25 °C). The samples should be kept in the dark to prevent algal growth.

If testing almost immediately after sampling (or sample preparation) is not possible, e.g. when preparing composite samples, cooling or freezing is recommended.

The most common and recommended way of preserving waste water samples is to cool to between 0 °C and 5 °C. When cooled to this range and stored in the dark, most samples are normally stable for up to 24 h (see ISO 5667-10). Cooling should commence as soon as possible after sampling, either in the field, for instance in cool boxes with melting ice, or in a refrigerator in the transport vehicle.

Deep freezing below −18 °C in accordance with ISO 5667-10 allows in general an increase in conservation. A few weeks up to 2 months, depending on the stability of samples, are generally the maximum storage periods.

Experience has shown that the quality of waste water can be affected during both freezing and thawing.

The use of biocidal preservatives should be excluded for the purpose of biotesting. The addition of highly concentrated acids or bases to stabilize the samples, e.g. HCl or NaOH, is not recommended either.

It should be stressed that, if there is any doubt, the chemical analyst and the biotester should consult each other before deciding on the method of handling and preserving the samples. If preservation techniques for the chemical analysis and for biotesting are not compatible, separate subsamples should be provided for the different purposes.

6 Apparatus and equipment

6.1 Selection of apparatus

Type, shape and material of the technical equipment are dependent on the test and nature of the sample. All materials which come into contact with the test sample should be such that interferences caused by sorption or diffusion of the test material, by elution of foreign matter (e.g. plasticizers) or by growth of organisms, are kept to a minimum. Inert materials are suitable, e.g. glass, PTFE. Tubing connections should be as short as possible and replaced from time to time. Contamination of the test material, e.g. by grinding grease from stoppers or fittings, should be avoided. Pipes made from copper, copper alloy or non-inert plastics are not suitable.

6.2 Silanization

In order to minimize adsorption of test material on containers, pipes, tubings, glassware or plasticsware can be silanized (siliconized) by soaking or rinsing in a 5 % mass fraction solution of dichlorodimethylsilane in chloroform or heptane. As the organic solvent evaporates, the silane is deposited on the surface, which should be rinsed many times with water or heated at 180 °C for 2 h before use. Silanization should only be used if highly adsorbable substances or water ingredients are to be tested and suitable inert material (e.g. PTFE) is not available.

6.3 Cleaning of apparatus and equipment

Prior to use, the apparatus and equipment should be cleaned with suitable cleaning agents, e.g. hydrochloric acid, sodium hydroxide, detergents, ethanol, sulfuric acid/ hydrogen peroxide and, where appropriate, sterilized, thermally or chemically (e.g. with hypochlorite solution). Chromosulfuric acid should not be used.

Repeated rinsing of the apparatus with distilled water (or water with the same degree of purity), ensures that no traces of cleaning or disinfection agent are left.

To efficiently remove traces of previous use, acid washing is recommended prior to final washing with distilled water.

7 Pretreatment and preparation of samples

7.1 General

The flow diagram (figure 1) contains information on commonly (but sometimes differently) used terms in biotest standards and guidelines.

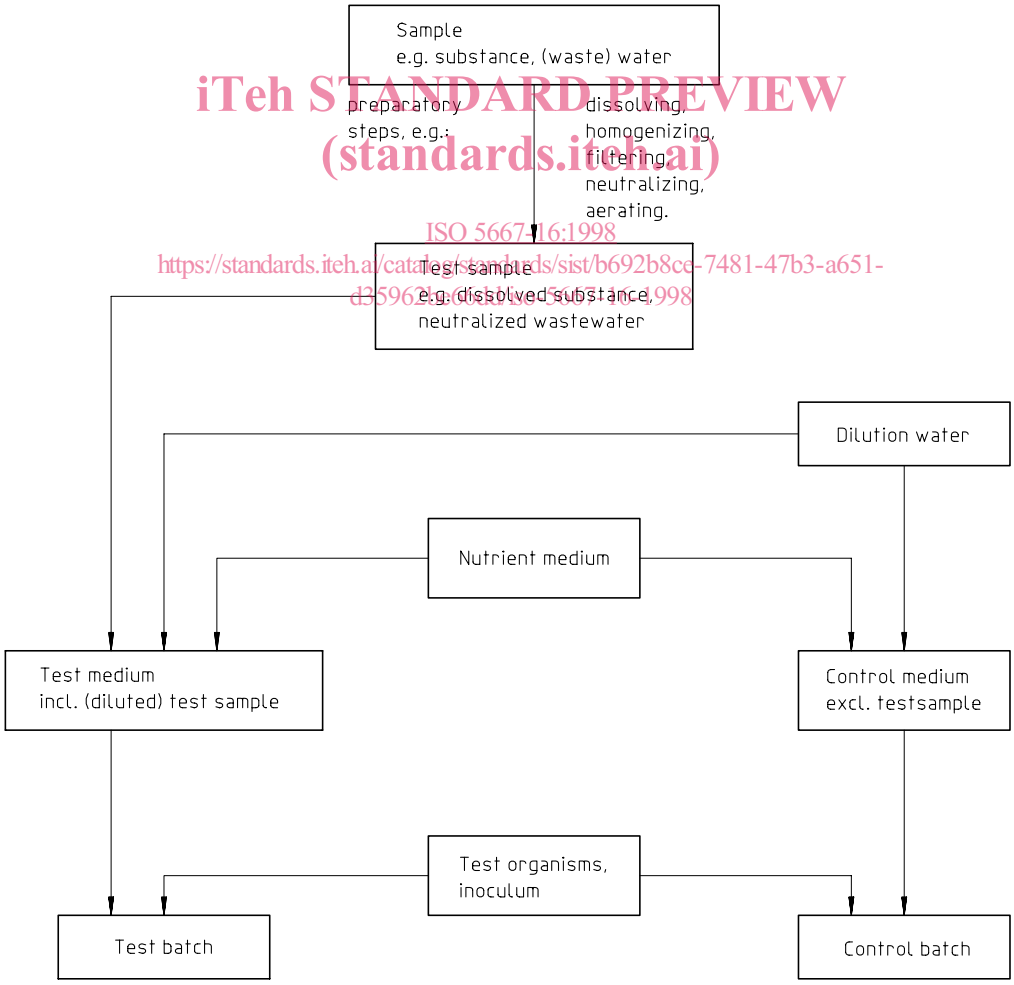

Figure 1 — Preparation of samples for biotesting

The sample, i.e. a chemical substance, is a preparation, solid or in solution, a mixture of various substances, water or wastewater. The test sample is made from the sample by means of various preparatory steps specific to the sample and the test, e.g. by dissolving, homogenizing, sedimenting, filtering, neutralizing or aerating. Dilution water is added to prepare a series of defined dilutions. Following addition of the test-specific nutrient medium, the test medium (including test sample) is obtained.

The final test batch is obtained by adding the test organisms – in the case of microorganisms called inoculum. The control batch, or in several parallels, the controls are prepared from a mixture of dilution water and nutrient solution with test organisms without the test sample.

When the effect or behaviour of a substance is known from previous tests ('reference substance') and when this substance is examined within the framework of a test series as test sample, this is called the reference batch.

7.2 Thawing

Samples stored frozen should be thawed immediately before use. Running water or a warm water bath at a temperature not exceeding 25 °C, together with gentle shaking, are recommended to avoid local overheating.

Complete thawing of the samples before use is essential, as the freezing process can have the effect of concentrating some components in the inner part of the sample which freezes last. Microwave treatment involves the risk of overheating.

7.3 Homogenization

An even distribution of all soluble and particulate components should be ensured. Gentle agitation, vigorous shaking, ultrasonic treatment or high-speed mechanical dispersion may be applied, depending on the nature of the samples. During this treatment step, attention should be given to the potential loss of volatile ingredients.

As a general rule, care should be taken that the original status of the sample be restored or at least be altered as little as possible.

7.4 Separation of soluble and particulate matter

In general, biotests are carried out with the original sample. In some cases, however, large amounts of particulate matter, sludge and sediment interfere with the behavioural requirements of test organisms (clogging of fish gills, impairment of filter feeding of daphnids, light limitation of algae).

If these deleterious effects are not intended to be reflected by the test results, such interferences can be avoided or overcome by various means.

Waters rich in particles can give rise to interferences, e.g. when quantifying by use of a particle counter. Microscopic counting is strongly impaired as well. Continuous dosing is rendered unreliable by clogging and blockage of tubing.

Filtration, centrifugation and other separation methods, however, involve the risk that active components, which are bound to the particles, are removed prior to the test. Moreover, problems related to filtration, e.g. adsorption on and leaching of filter materials, need to be taken into account. Sedimentation and centrifugation circumvent these problems. When carrying out tests in the presence of particles causing severe problems, it is recommended that the sample be allowed to settle for 30 min to 2 h or a coarse filtration (>50 µm) is carried out, thus removing only gross particles. The separated particle mass may be examined separately.

Some test methods offer the possibility of determining a correction factor for parameters such as turbidity.

Waters rich in bacteria interfere in tests related to bacterial activity, e.g. respiration inhibition. The interference due to the activity of bacteria in the sample can be accounted for, at least partially, by running suitable controls. When testing certain algae, eggs and fry or cell cultures, interference can be caused by bacterial infections. Available sterilization methods, such as thermal or UV-treatment or membrane filtration (0,2 µm), all involve a high risk of side effects. Glass-fibre filtration is preferable when filtering is necessary. Centrifugation, e.g. 10 min at 4500 *g* ± 1500 *g*, is, in general, preferable to filtration.

7.5 Preconcentration

Preconcentration of samples increases the concentration not only of harmful substances but of other water constituents as well, which probably can be deleterious in higher concentrations.

Furthermore it is essential to take into consideration that in any case the preconcentration is selective depending on the procedure applied. This alters the original composition pattern of water ingredients, e.g.

— liquid/liquid extraction with organic solvents and solid phase extraction by adsorption on solids (e.g. XAD-resins) are particularly efficient for hydrophobic water constituents. Ionic strength and osmotic pressure can be lowered. Toxic ions, polar chemicals and coefficient (e.g. masking) water ingredients, such as humic acids, can be excluded;
— evaporation and freeze-drying can lead to a loss of volatile substances and enhance the ionic strength and osmotic pressure;

— ultrafiltration can lead to a loss especially of small molecules penetrating the membrane.

The increase in concentration above the solubility threshold can lead to precipitation or flocculation of previously dissolved substances.

Bioaccumulation cannot be simulated by preconcentration of samples, since bioconcentration factors (BCF) cannot be related or extrapolated.

Certain ingredients of the water sample being concentrated can undergo chemical reactions at a higher rate than in the original sample.

Appropriate blank values can be obtained only if unpolluted reference samples, e.g. upstream of the contamination source, are available. The increase in salinity may be allowed for by preparing blanks with equal osmolarity and similar ionic composition (e.g. Na:K ratio).

It is not possible to extrapolate from acute tests with preconcentrated samples to chronic effects of the original sample.

Therefore it is preferable to choose a more sensitive test system or to prolong the exposure time rather than to preconcentrate a sample. If there is no sensitive method available to test the original sample and a pre-concentration procedure is applied, the result is the more contestable the higher the concentration factor.

For the above-mentioned reasons, tests for acute and chronic toxicity with pre-concentrated samples are generally meaningless and not recommended. In all cases test results obtained with pre-concentrated water samples should be interpreted with extreme caution. Preliminary investigations of this kind cannot be standardized and should be validated by further extensive investigations.

7.6 pH adjustment

The selection of the pH value to which the sample is to be adjusted is governed by the objective of the test:

— adjustment to the pH of the receiving water will produce results more representative of the effect of toxicants once in the environment;

— adjustment to a defined pH between 6 and 9 (which is usually tolerable for aquatic biota) will permit the expression of ionizable toxicants that would otherwise be masked by pH conditions outside this range.

Usually samples with extreme pH values exceeding the tolerance limits of the test organisms are neutralized. Neutralization should be omitted if the effect of the pH is to be reflected in the test result or if physical modification or chemical reactions (e.g. precipitation) are observed due to pH adjustment. The concentration of the acid or base required for neutralization should be such that the volume change is as small as possible. Passing the neutral point should be avoided.

The neutralizing agent should not undergo a reaction with the ingredients in the sample, which might, for example, lead to precipitation or complexation. Also it should not influence the test organism by enhancement or inhibition. Usually hydrochloric acid or sodium hydroxide solutions are recommended.

7.7 Preparation of stock solutions and test batches

7.7.1 Water-soluble substances

When preparing the stock solution, the weighed portion of the substance should not exceed the maximum amount that will dissolve (< saturation concentration). By means of stirring and/or heating, the solution kinetics can be enhanced. This should not lead, however, to substance loss or thermal decomposition of the test sample.

7.7.2 Emulsions and suspensions stable in water

In the case of emulsions (e.g. cutting oil emulsions) and suspensions (e.g. latex milk) that are stable in water, and also with substances forming these stable entities with water, graduated dilutions should be prepared.

If a homogeneous distribution is not obtained in the stock liquor, the mixture should be stirred or shaken for up to one day.

7.7.3 Poorly soluble substances

7.7.3.1 General

Substances with a solubility in water of less than approximately 100 mg/l should be considered as sparingly soluble. When examining poorly soluble substances, ensure that no undissolved matter remains as sediment, as floating particles or in dispersed form. Hence, in order to secure reproducible results, those methods are to be used that ensure the best homogeneous distribution of the test compound in the test batch.

In the case of toxicity tests, it is advisable first to ascertain whether the substance has effects in the range of its water solubility. It should be borne in mind that in the case of some pure substances there is sometimes overlapping between molecular-dispersed and micellar- and colloid-dispersed up to coarsely dispersed systems (example: isomer-pure surfactants). In the case of isomer mixtures, e.g. surfactants, there is no substance-specific solubility limit. Simple optical methods (e.g. light-scattering measurements) do not permit any reliable determination of the degree of dispersion.

It is impossible to recommend one single method for generating an optimum solution or distribution of the substances in the medium, since the method selected should correspond to the physical properties of the substance. For that reason it has to be left to the experience of the investigator and/or production information of the manufacturer to select an appropriate method.

The following guidance is derived from practical experience and contains advice on ways and means which enable the investigator, after weighing up the pros and cons, to select the most suitable method.

7.7.3.2 Testing in the water solubility range

For this purpose, a defined weighed portion of the substance (e.g. 100 mg) is mixed by stirring or shaking with 1 litre of distilled water approximately 24 h, preferably in the dark. The weighed portion for preparing the stock liquor shall be indicated. Following phase separation, the undissolved phase is fully separated by filtration (where necessary using a membrane filter, pore size 0,2 µm) or by centrifuging. The dilution series is prepared with the aqueous phase. Should it prove necessary, depending on the properties of the substance, (e.g. high viscosity or decomposition in water), shorter or longer mixing times should be considered, possibly involving the use of auxiliary agents.

NOTE Depending on the substances to be tested, centrifugation and filtration techniques can lead to different results.

If the solubility is reduced by adding media constituents (e.g. nutrient salts), it is advantageous to prepare a saturated solution by mixing the substance with the test medium. In individual cases, consideration should be given to replacing the salts (e.g. Ca or Mg ions) with others (e.g. Na or K ions), which do not precipitate.