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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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Contents

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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.

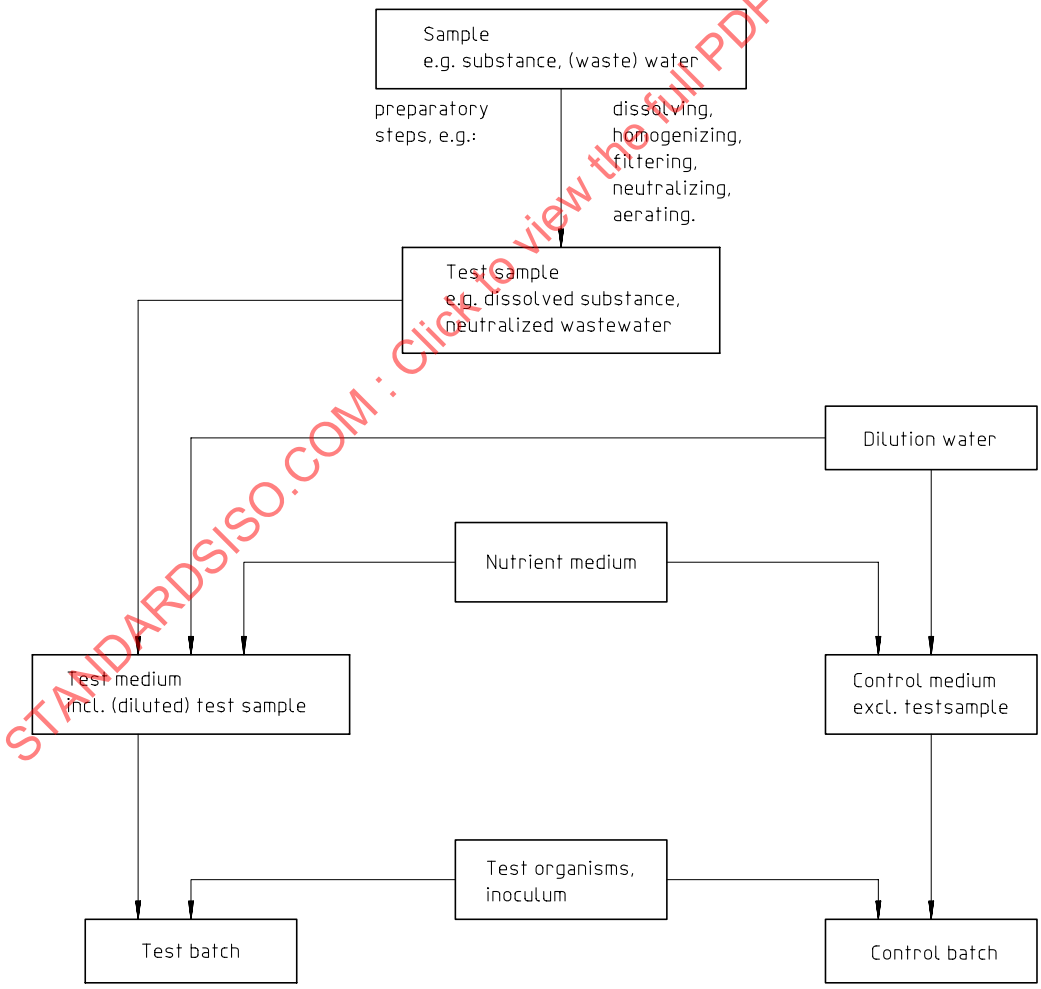

Figure 1 — Preparation of samples for biotesting

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.

A fine distribution of highly viscous fluids may be achieved by embrittlement at low temperatures (e.g. using liquid nitrogen) followed by mechanical crushing (e.g. in beater mills).

Filters for collecting of undissolved constituents shall be annealed (inorganic filters). Organic, e.g. polycarbonate, filters require repeated treatment in boiling distilled water in order to ensure that no constituents of the filter material are transferred into the test solution. Cellulose acetate filters are not recommended. Depending on the filter material, not only can filtration capture undissolved constituents; but also dissolved test substance can be lost through sorption. At concentrations <1 mg/l this can lead to considerable substance loss. The loss can be reduced by discarding the first portion of the filtrate. The use of inorganic filter materials, in general, will lead to lower losses than use of organic ones.

There is a variety of mechanical and chemical means to reach the saturation concentration. It should be borne in mind, however, that preference should be given to mechanical aids for preparing saturated solutions. Recourse should only be made to chemical aids (acids, bases, solvents) in exceptional cases.

Aids which can be used to reach the saturation concentration are:

a) *Sonication/high speed grinder* (advantage: higher dissolution speed; disadvantages: more difficult phase separation, possible decomposition and heating through energy input. Further details are given in ISO 10634.

b) *Temperature increase* (advantage: usually higher solubility and higher dissolution speed; disadvantages: enhanced volatilization, risk of decomposition and hydrolysis reactions, possibility of oversaturation with ensuing delayed precipitation. Saturated solutions should not be cooled.

c) *Use of acids/bases* with ensuing neutralization (advantage: exploitation of higher solubility of a protonated or deprotonated form; disadvantages: only applicable in exceptional cases, at extreme pH values risk of chemical change in the test sample, e.g. through hydrolysis, sometimes perhaps very slow establishment of equilibrium following neutralization, example: fatty acids/soaps).

d) *Dissolution of the substance in a volatile, nonaqueous miscible solvent* (e.g. *n*-hexane or petroleum ether) which is stripped after mixing with water (advantage: rapid fine distribution of the test substance; disadvantages: test substance can also be stripped off, low solvent residues in the test batch cannot be precluded).

e) *Dissolution of the substance in a water-miscible, nontoxic solvent* (e.g. ethanol, acetone, acetonitrile, dimethyl sulfoxide, dimethyl formamide). When selecting the solubilizer, its solubilizing capacity, toxicity, degradability and its volatility should be considered. The solubilizing agents (concentration: < 0,1 g/l) initially remain for a certain time in the test batch. It is unlikely that they lead to any major increase in the saturation concentration in water; they lead rather to the rapid fine distribution of the test substance in the stock liquor. An additional control batch with the maximum concentration of the solvent used in the test is necessary. Even if no damaging effects have been observed, it cannot be excluded that the presence of the solvent affects the action of the test substance on the test organisms, e.g. by aiding passage through the cell wall.

f) *Sorption of the substance on to an inert carrier*. A saturated aqueous solution can be prepared by applying the substance, where necessary, using a volatile solvent such as *n*-hexane or petroleum ether, to an inert carrier (e.g. glass beads, silica gel, chromatography resins). This is introduced into a flow-through system and rinsed with water. The dissolving substances are frequently available as a genuine molecularly dispersed solution.

7.7.3.3 Testing above the solubility limit

In degradation studies, poorly soluble substances are eventually tested above the solubility limit. It is important to achieve a high distribution rate for undissolved matter in order to ensure a large contact area between the microorganisms and the substance. It is always necessary to shake or stir the test batches throughout the test in order to guarantee a constant dissolution of the substance. The following methods are recommended.

a) Direct dosage

The substance is introduced directly into the test batch. Microscopic slides are suitable for introducing solid substances or highly viscous liquids. Water-miscible, non-toxic, undegradable solvents, e.g. dimethyl sulfoxide, are often used as solubilizer (see 7.7.3.2). This technique is restricted to non-volatile test substances.

b) Ultrasonic treatment

Treatment with ultrasound (e.g. 20 kHz, 30 min) can frequently lead to a sufficiently stable mechanical dispersion which, following approx. 15 min to 30 min settlement, can be distributed directly to the individual test batches. Suitable analytic methods, e.g. Total Organic Carbon (TOC) or specific analysis, are necessary to determine the initial concentration in the test batch.

c) Adsorption of the substance on to an inert carrier

This should be in line with the procedures outlined in 7.7.3.2 (adsorption of the substance on to an inert carrier). The carrier with the homogeneously applied test substance is now a constituent in the test batch. This method is not suitable for volatile substances, as they are eliminated during application and the subsequent drying of the carrier.

d) Dispersion of the substance with an emulsifying agent

Emulsifying agents in the concentration used (≤100 mg/l) should be non-toxic and stable throughout the test period. The following substances may be used as emulsifying agents:

— polyethene sorbitol monolaurate;

— polyethene sorbitol trioleate;

— nonylphenol-20 EO-acetal (well suited for degradation tests);

— modified castor oil (less suited for degradation tests);

— ethene oxide/propene oxide copolymers (suited for degradation tests).

In order to prepare a chemically stable emulsion, it is recommended that the test substance be mixed with the emulsifying agent prior to its introduction into water. Should an additional non water-miscible volatile solvent (e.g. *n*-hexane or petroleum ether) be used for the preparation of an emulsion, the pre-treated test sample should be stripped before the test organism is introduced.

Solid, poorly soluble substances are not usually tested above their solubility limit. Toxicity tests above the solubility limit are advisable only for readily dispersible solid substances and solid substances which are marketed as dispersions or come into contact with emulsifying agents when properly used.

Bioaccumulation studies and long term bioassays should not be carried out above the solubility limit.

The propensity of a substance to disperse in water is dependent above all on its density, its viscosity and its surface tension. By increasing the energy input, a lower particle size can be achieved and thus often a higher stability of the dispersion. There are, however, indications that the damaging effect of an emulsion is also influenced by the size of the emulsified droplets. Different pathways are likely to be available to organisms for the uptake of emulsified particles from that of dissolved substances.

Mechanical dispersions are often unstable. Phase separation can sometimes be avoided by constant stirring, shaking or other mode of mixing the test sample during the test. Care should be taken to prevent any mechanical damage to the test organisms.

NOTE Oil droplets and surface films can have deleterious effects on organisms, especially on daphnids. It is possible to preclude the contact of daphnids with surface films by mechanical separation by means of nets/sieves or by darkening of the surface.

The stability of an emulsion can be assessed by visual observation, chemical analysis (e.g. Dissolved Organic Carbon (DOC)/TOC; and by turbidity measurements. In principle, priority is given to mechanically prepared dispersions over dispersions with emulsifying agents.

There are two ways of producing a dilution series from a stock dispersion with emulsifying agents:

— maintaining a constant concentration of emulsifying agent;

— maintaining a constant concentration ratio of test substance to emulsifying agent.

As a rule, preference should be given to maintaining the same concentration of emulsifying agent in all test batches in order to ensure that the concentration-effect ratio depends only on the concentration of the test substance, thus avoiding the breakdown of the emulsion at higher dilutions. An additional control batch with the highest concentration of the emulsifier is always necessary.
When interpreting the results, it should be borne in mind that the effects observed are combined effects, even it the emulsifier itself shows no effect in the control batch.

7.7.3.4 Special problems with mixtures of substance or technical products

When testing substances with readily soluble toxic sub-ingredients (e.g. tetrabutyl tin contaminated with tributyl tin oxide) and poorly soluble substance mixtures (e.g. mineral oil products), the more readily soluble components can be enriched in the aqueous extract. This is often indicated by a rise in the DOC with increasing mass portion in the presence of an already existing undissolved phase. In order to record effects of this kind, it is recommended that an additional saturated aqueous solution be prepared and tested with a higher mixture ratio (e.g. 0,1 g or 1 g of substance per litre of water). Comparison with the findings obtained with various mixture ratios can indicate the effects of readily soluble components of a heterogeneous mixture.

As an alternative to this procedure, it is advisable to test substance mixtures which are not readily soluble and substances with readily soluble minor components by preparing a series of aqueous extracts in which the loading rates decrease geometrically. The aqueous extracts should be tested in undiluted form and the result referred not to the concentration in the aqueous phase but to the loading rates. It is important to note that the aqueous extracts should **not** be diluted.

As the chemical composition of the dissolved fraction of mixtures often varies considerably from that of the original product, it is advisable to examine also the effects of the original product in dispersed form (see 7.7.3.3).

8 Treatment of samples during the test

8.1 Aeration

The oxygen demand of test animals and heterotrophic microorganisms should be accounted for, e.g. by continuous or intermittent aeration. In special cases, e.g. waste waters with an extremely high biochemical oxygen demand (BOD), it is necessary to supply pure oxygen instead of air. Supersaturation (with respect to air) should be avoided. Problems of stripping of volatiles and losses of substances by foaming can be avoided by special methods (see 10.1.3).

8.2 Suspension

Particulate matter contained in the water may be suspended during the test if no interference is anticipated (see 7.4). Planktonic microorganisms should be kept in suspension during the test by appropriate methods, e.g. by shaking, stirring, rotation, aeration.

Care should be taken to avoid mechanical impairment of the test organisms.

8.3 pH adjustment and control

In certain circumstances (e.g. due to aeration) the pH will drift during the test even if the sample has been neutralized previously. The adjustment and control of the pH during the test should be decided according to the cause of pH drift and the objective of the test. Abiotic drift of pH can be distinguished by means of a batch without organisms.

From the analyst's and regulator's point of view it may be desirable to work with a substance in a clearly defined constant chemical status. Increase in pH value due to practical problems, e.g. stripping of CO_2 by aeration, should be avoided or counteracted.

This is especially important for the pH-dependent change of fish toxicity with ionized and unionized ammonia. Other examples for substances showing pH-dependent toxicity are sulfides, cyanides, amines, phenols and organic acids.

If, however, the change in pH during the test is an effect of fundamental vital processes such as photosynthesis in algal growth, which is necessarily accompanied by CO_2 consumption, it is reasonable not to adjust pH.

An allowed transition over a wide range would not only enhance the applicability and the indicative value of the test, but also offer the chance to trigger the effect of a chemical compound which otherwise might have remained undetected.

Increase in the pH value in algal assays can be reduced by various means, namely:

— addition of buffer;

— addition of carbonate;

— addition of gaseous CO_2;

— increased gas exchange by aeration and/or shaking;

— limitation of light;

— limitation of nutrients;

— limitation of temperature;

— limitation of test duration;

— reduction of inoculum.

All of these options have undesirable side effects and consequences, e.g. reaction with a test compound, precipitation, stripping of volatiles, complication of test design, limitation of growth, problems of measurability. In general, optimized gas exchange and conducting tests at the lower limits of light and temperature will reduce the problem.

Control of pH value in fish tests can be achieved by addition of carbon dioxide or hydrochloric acid or by conducting tests under specific atmospheric CO_2 concentrations. The pH adjustment during the test needs separate feedback regulation for each test vessel.

9 General guidance regarding test design

9.1 Preparation of dilutions

For physiological reasons, biological tests using aquatic organisms cannot be carried out using deionized water as the medium. Depending on the nature of the analysis, chlorine-free tapwater, synthetic fresh water or sea water (possibly with nutrient additives), ground or surface water shall be used. The latter is less suitable for toxicological but more appropriate for ecological investigations, e.g. to assess the load-bearing capacity of water in the light of the local situation (existing load, adaptation).

If a standardized synthetic medium is not used, it will be necessary to adjust the water hardness, the pH and, possibly, the specific molar ratio of calcium ions to magnesium ions when performing substance-specific toxicity tests. For some tests with marine organisms, the use of natural sea water is essential.

Defined quantities of the sample are combined with corresponding volumes of the dilution water in order to give the desired concentration or dilution.

The water specified in the appropriate standard is used as the dilution water. Usually, progressive concentration levels in geometric series are used.

9.2 Range-finding and limit tests

A dilution series is unnecessary when it is only desired to ascertain whether a given concentration or dilution level exhibits an effect (limit test).

It is expedient first in a preliminary range-finding test with fewer test organisms to determine the approximate range of effective concentrations, and only then to conduct the main test. Unstable samples (e.g. waste water) can change their properties during the time needed for the preliminary test. In this case the main test should be carried out immediately, possibly with rougher gradations over a wide concentration range.

9.3 Supplementary tests

When, in particular cases, the range of effective concentrations is not covered fully by the chosen concentrations, the test should be repeated or a supplementary test series be set up. In this case, at least two concentrations should be identical with two from the preceding test series. A joint evaluation of the two test series is only possible provided that the graduated concentrations show no larger variations than between the parallel batches within one series. Supplementary tests are not practicable in the case of waters and waste waters which are subject to alteration.

9.4 Control and reference batches

For each test, one or, where appropriate, several parallel control batches (9.5) should be examined. The reference or control batches should be identical with the test batch, but should not contain the ingredients to be tested. In order to identify possible interferences in the course of the test and to exclude unsuitable test organisms, it is recommended that a substance of known effect (reference substance) is tested at regular intervals. In the case of animal experiments requiring authorization, the number of parallel and reference batches should be kept to a minimum. Often-used reference substances in toxicity tests are e.g. sodium dichromate, 3,5-dichlorophenol.

9.5 Parallel batches/replicates

9.5.1 General

When testing replicates for each concentration/dilution level, it is possible to obtain independent observations under otherwise identical conditions. Marginal conditions such as temperature and light should be equal and constant as far as possible. In order to balance remaining differences, it is advisable not to arrange replicates side by side.

NOTE 1 Repetition of measurements: When undertaking several measurements of the same test batch (e.g. several countings from a single batch in the algal test), these are not truly independent observations (replicates). They rather serve to correct inaccuracies in the measurement. In such cases, it is possible to determine the mean value of the repetitions and use it as the 'better single value' in statistical estimates. In the case of the algal growth inhibition test, the parallel batches may, therefore, be viewed as true replicates, independent of the number of samples which were taken from them.

NOTE 2 Pseudoreplicates: If, e.g. in a Daphnia reproduction test, several animals are kept in one vessel, which means that the number of offspring produced can be determined only per vessel (and not per animal), the number of independent observations (replicates) corresponds to the number of vessels and not to the number of animals.

The number of parallel batches is often restricted because of shortage of time, space or financial resources. Furthermore, in the case of tests with vertebrate animals, it is imperative that the number of test animals be kept to a minimum for ethical reasons.

A further requisite for determining the number of parallel batches per concentration level and in control experiments is the kind of intended evaluation of the test (see clause 12).

9.5.2 Threshold concentrations (NOEC/LOEC)

In contrast to limit tests (see 9.2), threshold concentrations (NOEC/LOEC, see 12.3.1) are determined by an analysis of variance (ANOVA) (e.g. Dunnett's test) to recognize whether a specific concentration delivers a significant effect.

The number of replicates required depends on the endpoint variance, the number of and distance between concentration steps and the magnitude of the effect difference between test batch and control experiment, statistically verified at a given significance level. It is generally recommended that the number of control batches should be at least twice that of replicates per concentration/dilution level or increased by $\sqrt{p}$, where p is the number of test concentrations.

9.5.3 Concentration/response relationship

When determining the concentration/response relationship, the type of endpoint data, i.e. whether quantal (qualitative) or metric (quantitative) variables are determined, is of decisive importance for the test design.

The mortality (or immobility) of test organisms determined in the acute test is a typical quantal variable.

In contrast, metric variables show continuous increments, i.e. a response gradient. Typical metric variables are, e.g., body length or biomass, metabolic rates, oxygen production or consumption rates or enzymatic transformation rates. The number of young animals produced may also be regarded as an approximate metric variable.

In the case of metric variables, the results for the individual concentration/dilution levels are evaluated in relation to the control batches. Therefore special attention should be given to ensure the statistical reliability of the controls.

When determining the quantal variables, observations related thereto will be included directly in the concentration/response relationship. Therefore it is unnecessary for the number of replicates in the control experiment be higher than in the test batches.

In general, when determining the concentration-response relationship, it is advisable to reduce the number of parallels in test batches and increase the number of concentration levels. This is permissible because adjacent measurement points in a closely graduated dilution series can mutually support each other. Two replicates per concentration level are the minimum.

Adjacent concentration steps should not be established too close together, to avoid the effective concentrations being virtually identical due to the variability of the test system.

10 Special guidance regarding test performance

10.1 Problems and preventive measures for samples containing removable ingredients

10.1.1 General

Components of a water sample can be lost from the test system for various reasons:

— evaporation of volatile substances;

— biodegradation;

— abiotic degradation (e.g. hydrolysis, photolysis);

— sorption to or in vessel materials, particularly in the case of hydrophobic ingredients;

— foaming of surface-active agents;

— precipitation;

— flocculation.

In these cases, the substance fractions used in the test systems are not available for the organisms at a constant level throughout the test.

A preliminary test with or without test organisms can help to clarify which elimination path is responsible for the substance loss. Comparative measurements of concentration, e.g. of sum parameters, can reveal the loss mechanisms. Comparison of

— an open and a closed vessel gives an indication of evaporation losses;

— a sample exposed to and not exposed to light indicates the extent of photolytic degradation;

— an untreated and a silanized test vessel indicates sorption losses;

— a poisoned [e.g. with mercury(II) chloride or another suitable inorganic poison] and a nonpoisoned sample enables an estimation of elimination by means of biodegradation.

Substance loss during the biological test can, however, be merely due to adsorption to and accumulation in the test organisms or adsorption to food particles. In these cases, the organisms are still substantially exposed, although only a fraction of the substance can be determined analytically in water. It can be clarified whether real or simulated substance losses occur, by means of comparative analyses of batches with or without organisms and feed.

There are indications that in the case of microorganisms (e.g. algae, bacteria) the sensitivity of the test system decreases with the increase in the organism density. Loss of substances can be compensated by subsequent dosage or, better, by means of semistatic or flow-through systems to avoid accumulation of metabolites in the test system.

There are limits, however, in the case of poorly soluble substances, from which sufficiently concentrated stock solutions cannot be prepared.

10.1.2 Volatilization

Particularly in test methods requiring aeration, volatile substances are rapidly stripped from the test system. In such cases, the use of closed or flow-through test systems should be considered. It should be borne in mind that, for instance in the case of the cell multiplication inhibition test with bacteria or algae, a sufficient exchange of gas should be guaranteed.

In the case of volatile toxic substances, it should be ensured that there is no risk to the personnel conducting the test.

10.1.3 Foaming

Surface-active substances accumulate on the surface of a liquid and tend to form bubbles when the test batch is aerated.

By increasing the surface:volume ratio (flat test vessels) or, where appropriate, by using a ventilator to blow the surface, the necessary oxygen supply can be ensured without foam forming.

The use of antifoaming agents leads to unpredictable interaction with the test substance and should generally be avoided except in special cases (e.g. biodegradation studies).

10.1.4 Adsorption

Hydrophobic substances can be adsorbed to vessel walls and then – especially at low concentrations – are no longer fully bioavailable. In order to prevent large losses of substance, it is recommended that the vessels be incubated for at least 30 min with the sample at the envisaged concentration prior to the test, the sample then be discarded, and the vessel subsequently filled with fresh sample in order to prepare the test batch.

A silanization of the vessels can reduce the sorption capacity of the wall surface. It can, however, only be recommended if no other inert material is available and if it can be ensured that no residual silicone is leached (see 6.2).

10.1.5 Precipitation/flocculation

Precipitation of test material can occur during the test, due to reactions with constituents of the nutrient medium (e.g. calcium ions) in particular when the pH shifts in the test batch. Measures to maintain a constant pH (see 8.3) can help to avoid this interference.

10.1.6 Degradation

Ingredients can undergo different types of degradation, namely biological, hydrolytic or photolytic, during the test. This can lead to the formation of secondary products (metabolites) whose toxicity is different from the original product.

It is often difficult to prevent biodegradation in static test systems, as the test organisms such as fish and other test organisms cannot be separated from their accompanying bacteria.

In certain cases, e.g. in the fish test, a lower test temperature leading to decreased biodegradation may be applied if the fish species can be changed (e.g. rainbow trout instead of zebra fish).

In bacterial tests, biodegradation is often inherent in the system and reflected by enhancements with regard to the test criterion (e.g. oxygen consumption rate, cell proliferation). In certain cases, flow-through systems may be used. Thermotraps can normally prevent the spread of bacterial infection back through the tubing.

10.1.7 Hydrolysis

Some ingredients (e.g. isocyanates, esters, anhydrides) hydrolyse in water, which means that in the course of the test the organisms are increasingly exposed to decomposition products. In the case of hydrolysis, the pH value of the test batch can be altered, which sometimes leads to changes in the rate of hydrolysis. Under these circumstances, the pH should be adjusted or the buffer capacity increased. In individual cases, a lower test temperature, leading to a reduction in hydrolysis rate, may be applied by selecting other test species. When using flow-through or semistatic test systems, the effect of the undecomposed sample can be examined more easily.

In the case of biodegradation tests, hydrolysis of the test material can result in a shift of the pH-inhibiting ranges during the test. When the pH cannot be controlled by buffers or adjustments, it is recommended to prepare an aquatic stock solution, allow hydrolysis, e.g. for 24 h, neutralize the solution and start the biodegradation test with the hydrolyzed sample.

10.1.8 Photolysis

Some ingredients (e.g. hexachlorocyclopentadiene, EDTA, hexacyanoferrate) are decomposed through exposure to light. In the case of algal tests, the effect of light is inherent in the system.

In other tests, such decomposition reactions may often be reduced or avoided by working in a darkened environment (where necessary using red light). As with biological and hydrolytic degradation, it is preferable to carry out the testing in flow-through and semistatic test systems.

10.2 Problems and preventive measures concerning coloured and/or turbid samples

In some biological tests, endpoint determination is based on a spectrometric measurement (photometry, fluorometry). In the case of highly coloured or turbid samples, the inhibiting effect produced cannot be determined reliably. The following steps may be taken to overcome this situation:

— different method for endpoint determination (e.g. cell count instead of turbidity measurement in the algae test);

— measurement of turbidity caused by the organisms by using a different wavelength or two different wavelengths (dyes often have wavelength characteristics different than the light scattering caused by microorganisms);

— combination with another suitable method, e.g. measurement of oxygen consumption or oxygen production rate at the end of a cell multiplication inhibition test; in this case the administration of nutrient medium should be renewed;

— determination of the influence on the result of the dye and/or turbidity with the help of combined measurement/test vessels in which the test sample and organisms are separate from each other (e.g. colour correction cell in the luminescent bacteria test).

In the algal test, coloured and turbid substances can directly impede cell growth as a result of light attenuation; this should not to be mistaken for toxic effects. The following are suitable for differentiating between toxicity and physical interferences of coloured substances in the algal test:

a) testing at different light intensities as the growth rate above light saturation is almost independent from the light intensity;

b) reducing the light path by reducing the depth or the volume;

c) measuring the reduction of cell proliferation in a control batch when the light previously passes through a liquid light transmission filter, e.g. a flat dish containing corresponding dilutions of the sample with the corresponding colour and layer thickness;

d) utilizing a light-transmission filter by preparing dilutions reciprocal to the exposure concentration, thus maintaining a common light-pathlength (constant depth) and the spectral absorbance characteristics of the sample.

11 Special biological assays

11.1 Testing for biodegradation

The experimental determination of degradability of substances and waste water and bioaccumulation studies are also biological tests. Degradation can be considered as the decomposition of an organic compound into simple constituents (metabolites) through physicochemical effects (e.g. photolytic degradation by light) and/or biological activity (e.g. biological degradation by microorganisms).

A distinction is made between:

a) primary degradation by means of which the substance loses specific properties (identity, activity) and breaks down into simpler constituents;

b) ultimate degradation, i.e. full degradation into thermodynamically stable inorganic products, e.g. carbon dioxide and water. This leads to the creation of new biomass in addition to carbon dioxide, water and mineral salts.

The criteria for biodegradation are, e.g.

— the reduction in the reaction components (e.g. O_2) necessary for biodegradation;

— the increase in the reaction products arising from total degradation (e.g. CO_2);

— the transformation of ^{14}C-labelled substance into $^{14}CO_2$;

— the reduction in the initial substance detectable by means of specific analyses;

— the reduction in the substance detectable by means of nonspecific sum parameters (e.g. TOC, DOC).

NOTE 1 The two last statements give no indication of whether this is biodegradation or physicochemical elimination (e.g. through-stripping) or whether chemical structural changes (e.g. through primary degradation) have taken place.

A distinction can be made between elimination through sludge sorption and biodegradation when the (potentially adapted) sludge is used at the end of a test for inherent (potential) degradability as the inoculum for a test for ready degradability, when in parallel a poisoned batch is examined in which no biological processes are taking place or when the occurrence of metabolites can be proven analytically.

Biodegradation test methods can be classified in the following groups:

a) *Methods to measure ready biodegradability*:

When measuring ready biodegradability, the test substance is brought into contact with a small quantity of, polyvalent microorganisms. The tests are designed as discontinuous tests, i.e. they involve single-substrate inoculation. The course of biodegradation can be observed by means of various parameters. When substances fail to meet the minimum degradability criteria in this test system, their degradability can be examined in a more extensive test system.

b) *Methods to determine potential (inherent) biodegradability*:

In methods to determine potential biodegradability, a distinction is made between batch and semicontinuous test systems. Both have a relatively high inoculation density with polyvalent microorganisms.

The batch systems are designed to work with single inoculation and substance administration, whereas the test substance in the semistatic system is administered on a daily basis.

NOTE 2 The use of the terms 'ready' and 'inherent' biodegradability as defined in OECD guidelines should be restricted to testing according to OECD guidelines. For further details see ISO 15462.

Biodegradation can further be determined by:

a) *Simulation tests*

Simulation tests serve to determine biodegradability under environmentally relevant conditions. Validated models are required depicting these environmental conditions. The best models so far are the simulation models for sewage treatment plants in which the degradation of a test substance is observed under flow-through conditions.

b) *Other methods*

These include e.g. test methods in which the batch contains sediment, and anaerobic methods in which the conversion of the test substance into CO_2 and CH_4 is observed.

c) *Measurements*

Basically, a distinction is made between parameters covering the mineralization of a test substance (measurement of oxygen consumption, measurement of CO_2 release) and parameters recording the disappearance of the original substance and/or a characteristic property of the original substance, e.g. surface-active effect in the case of surfactants. In individual cases, e.g. when testing pesticides, tests will focus not only on the degradability of the substances themselves but also on the main metabolites in soil, water and in plants. When testing the degradability of substance mixtures and preparations, it should be borne in mind that no statements can be made on the degradability of minor constituents and additives.

Further details on execution and evaluation of degradation tests can be found in e.g. ISO 7827, ISO 11734, ISO 11733, ISO 10707, ISO 9408, ISO 9439, ISO 9887, ISO 9888, ISO 10634, ISO 10708, ISO 14592, ISO 14593, ISO 15462.

11.2 Testing for bioaccumulation

Bioaccumulation can be considered as the accumulation of substances in living organisms from the environment or via food. The accumulation of substances in the food chain is referred to as biomagnification. The bioaccumulative processes lead to end concentrations of substances in aquatic organisms possibly exceeding the starting concentration by several orders of magnitude.

The bioaccumulation of chemical substances is influenced by substance properties such as lipophily, water solubility and molecular size/structure, by the stability of chemical substances in water and by the metabolic activity in the organism.

The criterion for bioaccumulation is the bioconcentration factor (BCF). It describes the quotients of the concentration in the organism (c_1) and the concentration of the substance in the medium (e.g. water) or another parameter (e.g. food) (c_2) at specific times or under steady-state conditions (BCF_{st}).

$$BCF = \frac{c_1}{c_2} = \frac{k_1}{k_2} \quad \ldots (1)$$

where

k_1 is the uptake rate;

k_2 is the elimination rate.

A plateau or steady state has been achieved when three subsequent analyses at intervals of at least two days do not differ by ± 20 %.

A preliminary estimation of the bioaccumulation potential is possible by means of the partition coefficient *n*-octanol water, $\log_{10} P_{ow}$, which is a measure for the lipophilic property of a substance [41]. In the range of $\log_{10} P_{ow}$ 3 to 6, a relation of BCF = 0,1 P_{ow} is often observed for fish.

The $\log_{10} P_{ow}$ is not suitable to estimate the bioaccumulation potential in the case of

— ions (e.g. heavy metals);

— surfactants (underestimation);

— substances with molecular weights MW >700 (overestimation due to unavailability of excessively large molecules);

— highly lipophilic substances ($\log_{10} P_{ow}$ >6) (overestimation since the linear relationship no longer holds).

NOTE Accumulation experiments with highly lipophilic substances can give erroneous results or are precluded per definition because the steady-state conditions have not yet been established and/or the analytic determination of the substance dissolved in water remains uncertain.

In general, results of bioaccumulation experiments are more affirmative than estimations by means of $\log_{10} P_{ow}$ relations. BCF determinations should not be carried out with substance concentrations in the range of toxic effects to the organisms. Furthermore, the range of water solubility should not be exceeded.

In fish the substances will usually be incorporated via the gills and through the skin. An ingestion with the feed is relevant in most cases only for highly lipophilic substances ($\log_{10} P_{ow}$ >5 and mostly >7).

For a more elaborate assessment of the bioaccumulation potential, not only the BCF but also

— the depuration kinetics,

— the height of the residue plateau, and

— the distribution in specific organs

are indicative factors.

If the elimination follows a one-compartment model of first order kinetics, it is possible to compute a CT_{50} value (clearance time for 50 % elimination) as follows:

$$CT_{50} = \frac{\log_e 2}{k_2} \quad \ldots (2)$$

Often a two-phase excretion is observed, i.e. a rapid elimination followed by a phase of slower elimination due to different uptake rates in specific organs, metabolic processes or different excretion pathways. For investigations of this kind, radiotracer techniques are most suitable.

The generally very low test concentrations in an accumulation test (1/100 or 1/1000 of LC_{50}) impose special requirements on the efficiency of the analytical methods accompanying the test. In some areas (transformation of test substances) radio-labelled substances may be used.

Further details on determining bioaccumulation can be found in the OECD Guideline 305.

11.3 Testing for genotoxicity

Genotoxicity/mutagenicity tests serve as preventive measures within an overall public health maintenance system. At the present time there is little reliable knowledge about the effects of mutagenetic substances on aquatic ecological systems.

Genotoxicity can be considered as an alteration of the cellular genome under the influence of substances. As far as these changes are transmitted to the next cell generation, it is possible to speak of a mutagenic, genotype-changing effect. Genotoxic effects play an essential role in the aetiology of cancer. Not all genotoxic alterations, however, are inherited by the daughter cells, because cells have compensatory repair systems for deoxyribonucleic acid (DNA), which are capable of reversing many adverse alterations. In addition, drastic effects often lead to cell death. The induction of repair systems can be viewed as proof for the occurrence of genotoxic effects. Mutations are divided into three types.

a) Gene mutations in which the base sequence in a gene is changed through the exchange of a DNA base (point mutation) or through the introduction or withdrawal of one or more DNA bases (changes in the reading code, frame shift mutations).

b) Chromosome mutations in which the visible chromosome structure is changed, e.g. through deletion, translocation or inversion. Chromosome mutations can lead to the formation of deletions recognizable as micronuclei. The chromosome structure can be especially well perceived during the metaphase of cell cycle.

c) Genome mutations in which the total chromosome quantity of cells is changed. In this type, the quantity of the individual cells (aneuploidy) or the entire chromosome set (polyploidy) can change.

Chromosome and genome mutations can only be observed in cells of eukaryotic organisms. *In vivo* and *in vitro* test procedures are distinguished. *In vitro* procedures are sensitive to demonstrate the genotoxic potential of a test batch and they are appropriate to minimize animal tests. Because, however, *in vitro* procedures constitute sub-organismic test systems, their informative value is limited.

The mechanism of the genotoxic effect can be differentially ascertained by means of *in vitro* procedures. As a general rule they are carried out with cell cultures from mammals (e.g. CHO cells, V79 cells). Their metabolic potential for xenobiotics is comparatively low. For this reason microsomal enzymes (S9 fraction), e.g. from liver tissue induced by polychlorinated biphenyls (PCBs), are added (metabolic activation) as parallel test series. Although S9 fraction can cause a reduction in activity with some substances, it can, however, induce first a mutagenic effect with numerous other substances (promutagenic) through biotransformation.

The S9 fraction is also added in bacterial (prokaryotic) test systems (e.g. in the Ames testing procedure) in a parallel test series. With bacteria, genome-altering potentials can be quickly and easily identified. Nevertheless, an extrapolation of the results to higher organisms poses significant limitations.

When carrying out bacterial tests and eukaryotic *in vitro* procedures, it is expedient to work under sterile conditions. As water, and especially waste water, samples very often contain many bacteria, samples of this type generally should be filtered through a membrane filter prior to their use in most of the test systems. The concentrations/dilutions used should not be cell damaging (cytotoxic). Reference batches (positive controls) to verify the reactivity of the cells should be simultaneously prepared.

12 Evaluation

12.1 General

Evaluation of the test results first involves the critical inspection of data and a presentation and description of the test results using graphs, tables and suitable statistical parameters, e.g. mean values and variations (descriptive statistics).

In many cases, this is followed by more extensive statistical processing which aims to determine concentration- or dose/ response relationships, to calculate suitable statistical parameters for the quantum of action and to examine the statistical significance (estimating and testing statistics). This more extensive statistical evaluation is useful only if the data are sufficient for this purpose. This requires first of all critical examination of the data.

12.2 Basic data inspection and description

Every evaluation should begin with a critical examination of the test results obtained. All data, primary readings or transformed data deduced from the measurements, are checked for plausibility, especially in the case of outliers.

NOTE Outliers should be eliminated by the evaluator only after expert judgement. Outlier tests may be used here as mathematical aids.

A graphic presentation, preferably of the untransformed values, is helpful in considerations on plausibility. Often a semilogarithmic graph of the effects measured is particularly suitable. Any graph should clearly present the concentration units, the number of controls and parallel batches and the effect parameters.

Furthermore, a graph provides valuable information on the behaviour of test compounds and the extent to which the determination of a mathematical concentration/response relation is advisable. It can also indicate which statistical model is likely to be most suited.

In the case of the more advanced evaluation, priority should, in principle, be given to arithmetic methods over purely graphical methods as they usually permit the calculation of variances and confidence ranges. Data should be presented in tabular form together with the mean values calculated, the variation coefficients and the number of independent observations from parallel batches (replicates).

In some cases in which simple, defined parameters, e.g. threshold dilutions, are to be reported, there is no need for a more extensive statistical evaluation. In these cases, the evaluation ends with the calculation of percentage inhibitions and the decision whether a measured effect is higher or lower than a given limit value.

12.3 Statistical evaluation

12.3.1 General

The more extensive statistical evaluation aims

a) to present a mathematical relation between the effect measured and the concentration used (concentration/response relationship),

b) to calculate statistical parameters which characterize the effect and

c) to carry out statistical tests or to give confidence ranges (for example refer to ISO 8466).

It is customary to give concentrations as statistical parameters for the effect of a substance at which a specific quantum of action was observed (EC: effective concentration, EC_x; x: x % effect).

For example, the EC_{50} gives the concentration at which the effect can be observed in 50 % of the test animals or 50 % effect (e.g. mortality, immobility) (mean effect concentration, 50 % quantile). These parameters for the quantum of effect are derived from the concentration/response relation.

In some tests an effect concentration is determined, upwards of which a statistically significant effect was observed, e.g. LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration). These parameters can be determined by comparing the test results from various concentration levels with control results using statistical tests for the analysis of variation (ANOVA) or, if their prerequisites are not fulfilled, non-parametric methods (e.g. Multiple U-tests).

NOTE Such a figure, by definition, will be one of the tested concentrations. Alternatively, the LOEC may be a point estimate, EC_x. The value of x is determined from practical experience with the particular biotest method. In many situations an EC_{10} will be an appropriate LOEC estimate. For certain test methods with larger variability or more generally in situations where the confidence limits around EC_{10} are larger, the EC_{10} may be replaced by an EC_{20}.

The decisive factor in selecting a suitable statistical evaluation or test method is whether the test criterion was related to the quantal (qualitative) or metrical (quantitative) type of variables (see 9.5.3).

12.3.2 Quantal (qualitative) variables

The methods listed here are suitable for biotests in which the variable "mortality" or "immobility" is being studied (e.g. acute toxicity tests with fish and daphnia). The classic estimation method for quantal variables of this kind is based on the maximum likelihood principle. In the case of normal distribution this leads to probit analysis [30], [31], with logistic distribution to logit analysis [32] and using the Weibull distribution to Weibit analysis [33]. In the case of quantal variables, parameter estimates using the maximum likelihood principle take into account the variance heterogeneity of the measurement values. This constitutes a major difference to an unweighted regression following linearisation of the concentration response curves (e.g. using probit paper). In this situation, this does not lead to valid confidence ranges.

12.3.3 Metric (quantitative) variables

The methods of probit, logit or Weibit analysis mentioned above are not suitable for metric (quantitative) variables. As a rule, the least-squares principle of linear and non-linear regression is used for the estimation methods. The aim is to select a function which allows the best adjustment to the data (lowest residual variance). If the measurement values reveal different variances for the individual test concentrations, the regression analyses should be conducted with suitable weighting factors [34].

It is not recommended to relate the effects measured in the test batches to the untreated controls and to enter them against the logarithms of the concentration. This transformation can lead to heterogeneity of the variances and, therefore, comprises major disadvantages in respect of the statistical evaluation. For this reason, the evaluation should always be made with non-transformed data.

The effect concentrations are calculated, where possible, from the concentration response functions. In the case of quantal variables (e.g. from the acute fish or daphnia test), it is customary to give the EC_0, EC_{50} and EC_{100} which can also be described as LC_0, LC_{50} and LC_{100} (LC: lethal concentration).

They stand for:

- EC_0: highest tested concentration at which no effect is observed in line with the test criterion (LC_0: all organisms survive);
- EC_{50}: concentration at which there is an effect on 50 % of the organisms in line with the test criterion (LC_{50}: 50 % of the organisms are dead);
- EC_{100}: lowest concentration at which there is an effect on 100 % of the organisms in line with the test criterion (LC_{100}: all organisms are dead).

The EC_0 and EC_{100} are parameters derived directly from the experiment and not statistically determined threshold concentrations. NEC (No Effect Concentration) is derived from a concentration/effect relationship extrapolated to zero.

In the case of metric variables, the EC_{50} is usually calculated, sometimes the EC_{10} as well (e.g. in the algal growth inhibition test). It should be borne in mind that for example the EC_{50} does not correspond to a mean effect concentration, as in the case of the quantal variables, but designates that concentration at which a metric variable is on average 50 % lower than in the control.

NOTE IC (inhibitory concentration for *x* % effect). The designation EC_{50} is sometimes also used in cases in which a variable is inhibited by 50 % in relation to the control (e.g. number of young animals in the daphnia reproduction test, biomass and growth rate in the algae inhibition test). In order to distinguish these effect concentrations determined from metric variables from those mentioned above, some authors suggest that they should be described as IC_x (inhibitory concentration for *x* % effect).

The confidence interval can be calculated using the Fieller method [30], [36], [38].

If the data situation is such that none of the above-mentioned methods can be applied, the EC_{50} and its confidence range can be approximated using alternative methods e.g. (Moving Average Method, Trimmed Sperman Kärber Method [30, 31] or simple interpolation (if necessary from EC_0 and EC_{100}). If EC_{100} is not obtained with undiluted samples, the maximum EC obtained should be reported.

In many biodegradation tests the number of measured degradation values in the plateau phase is not sufficient to allow a statistical treatment. The last result of the plateau phase is, however, often not representative for this phase. In such cases it is recommended to indicate the test result in a 10 %-range, e.g. biodegradation degree 70 % to 80 % DOC removal.

12.3.4 Statistical confirmation of results

12.3.4.1 General

The statistical tests mentioned here are used to determine whether in general the tested substance has a significant effect and/or from which concentration upwards a significant effect is observed. This concentration is described as FOEC/LOEC (First/Lowest Observed Effect Concentration), and the next lowest concentration (no significant effect) as NOEC (No Observed Effect Concentration).

These parameters depend on:

— the variance of the measured parameter;

— the number of parallels in the test concentrations and controls (replicates);

— the number of concentrations tested;

— the increments of the concentration levels.

Thus, the result is influenced to a large degree by the test design. Here, too, the choice of a suitable statistical method depends on whether quantal or quantitative (metric) variables are examined.

12.3.4.2 Quantal variables

The methods mentioned here are suitable for tests in which the variable "mortality" or "immobility" is examined (e.g. acute toxicity test with fish and daphnia). In order to statistically confirm the effect observed, tests for nominal data scales are suitable. In order to determine the LOEC/NOEC, the binomial test according to Fisher is suitable.

12.3.4.3 Metric variables

The choice of a suitable test method depends on whether the test criterion (e.g. biomasses, growth rates, production of young animals and metabolic rates) follows an approximated normal distribution and whether variance is homogeneous. Then, the parametric statistical methods can be used which are more reliable.

In the case of normal distribution and variance homogeneity, statistical confirmation of the substance-specific effect is undertaken using simple variance analysis (ANOVA). NOEC and LOEC are determined using the Dunnett or Williams test [35].

If these preconditions are not fulfilled, the effect can be confirmed using the Kruskal-Wallis test. In that case, multiple U tests (e.g. according to Bonferroni-Holm [39]) are suitable for determining the LOEC/NOEC.

NOTE 1 The EC_0 cannot be equated with the NOEC. The former is determined directly from the data without using a statistical method. Both, however, are determined to a major degree by the design of the test (e.g. number and magnitude of the concentration increments).

NOTE 2 The determination of the NOEC using the above-mentioned statistical tests is currently being critically discussed [40]. Alternatively, it is proposed to determine the effect threshold concentration NEC from the concentration response relationship [71].

13 Presentation of results

13.1 Toxicity tests

Toxicity tests mainly give the effective or threshold concentrations, e.g. NOEC/LOEC, EC_{10}, EC_{50} and EC_{90} or LC (lethal concentration), respectively, where appropriate. The concentration data should clearly indicate whether these are based on the effective ion, compound or e.g. dilution step.

Where possible, the concentration response curve should be presented together with the test results in a graph, e.g. as shown in figure 2, where the test results and EC_x values are presented. This offers a clear picture of the character of the concentration response relationship and the agreement of the results in relation to the model.

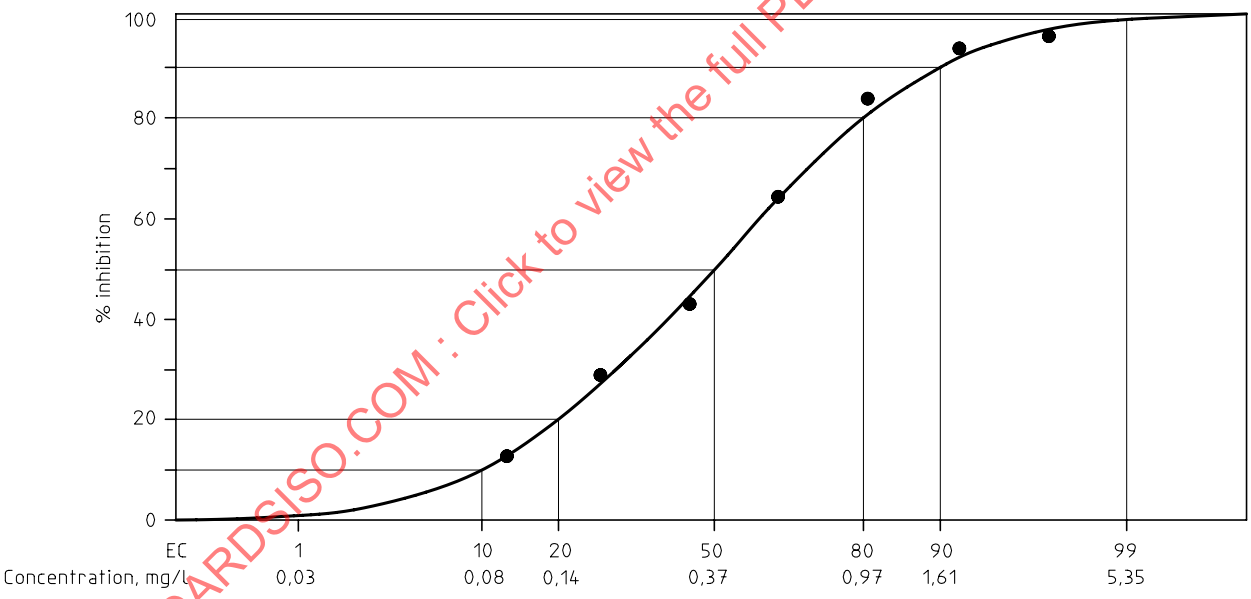

Figure 2 — Example of concentration/response curve adapted to normal distribution for inhibition

The test criterion ('endpoint'), test organism, exposure time and, where possible, the confidence ranges, e.g. for the EC_{50}, should also be given (see 12.3.4).

NOTE When testing waste waters by means of graduated dilutions, the lowest ineffective dilution may be reported (see annex A).

13.2 Degradation tests

The degree of degradation is described by the percentage decrease in concentration of one (or several) parameters as mentioned in 11.1.

The degradation kinetics (lag phase for adaptation, half-life time, '10 days window') should be described.

13.3 Bioaccumulation tests

The time course of substance concentration in the whole body or special tissues should be described by means of graphs or tables in order to demonstrate that a steady state has been achieved. The BCF should be reported as a dimensionless figure rounded to not more than three significant figures.

The basis of BCF (e.g. whole body, organ, fresh or dry mass) and its experimental design (static, semistatic, dynamic) should be stated.

13.4 Genotoxicity tests

In general the number of observed incidents (e.g. chromosomal aberrations) or acquired metabolic capacities (e.g. induction of repair enzymes) are reported in relation to given concentrations of a substance or dilutions of a water sample. This value is related to a corresponding value of a control batch. The value may be normalized according to the cell number at the end of the test. The concentration/effect relationship may be evaluated according to clause 12. If substances are tested, the result is generally reported qualitatively (positive/negative).

NOTE If waters are tested, the result may be reported as lowest ineffective dilution.

14 Test report

The following data should be recorded in the test report:

a) name of the laboratory performing the test;

b) date and period of test;

c) reference to the test method (International Standard number) used;

d) test organism (e.g. scientific name, strain, source), therapeutic or acclimatory pretreatment (if any);

e) designation of test material (batch number, origin, date and period of sampling);

f) sample pretreatment (e.g. preservation, preconcentration, homogenization, pH adjustment, type of neutralizing agent, pre-aeration);

g) data, derived, condensed or transformed, including, if appropriate, results of positive controls (reference batch);

h) chemical and physical data determined during the test (e.g. temperature, CO_2-content, pH, turbidity, precipitation, possible change in substance concentration, etc.);

i) any deviation from the test protocol (nature of dilution water, nutrient solution, aeration, temperature, etc., number of organisms or density of inoculum, number of parallel batches and controls);

j) evaluation method (logit, probit, graphical, computational);

k) details of the test results;

l) commentary on the test results, if necessary;

m) signature of responsible investigator;

n) signature of quality controller, if appropriate.

15 Basic principles of quality assurance for biotesting

15.1 General

The term 'quality assurance' covers all measures taken in order to state and report the quality and potential errors in test results. Most measures in a series of International Standards (ISO 9000 series, etc.) introduced under the term 'analytical quality assurance' may be applied to biological test methods as well.

The quality assurance of a test includes the planning of sampling, measurements and evaluation, documentation and assessment of the results and their recording.

In line with the importance of quality assurance in the procedure, a corresponding programme should be incorporated adequately into the structure of the overall organization considering that sometimes, depending on the test method used, up to 30 % of the laboratory time needs to be devoted to achieving adequate quality assurance.

The quality assurance measures can be subdivided into four sections:

— preparatory phase;

— internal quality control;

— external quality control audit;

— evaluation and documentation.

15.2 Preparatory phase

15.2.1 Organization and staff in the test laboratory

Users of this guide should try to ensure the following.

The test laboratory is organized to allow a sufficient number of staff with the necessary initial and additional training and experience in order to carry out the tasks assigned.

Normally, the general manager of the test facility is responsible for the organizational and administrative sides and, among other things, for ensuring that the requirements related to staff and equipment for conducting the tests are fulfilled.

The operation and performance is supervised by a biotest supervisor, who is responsible for seeing that the test is correctly and properly carried out and documented in a test report.

The quality assurance tasks involve the verification of compliance of the test performance with the methods and criteria laid down in the International Standard and whether the raw data are correctly recorded and correspond to the results given in the report.

In larger laboratories, it may be advisable to assign a person with the necessary qualifications to monitor quality assurance on a full- or part-time basis. The tasks may be assumed by an independent organizational unit.

Regular and adequate continuing training is provided both for the laboratory manager and other members of the laboratory staff.

15.2.2 Laboratory equipment

Users of this guide should ensure the following.

The test laboratory has the appropriate equipment for conducting the tests. In addition to the technical equipment, the laboratory should have appropriate building facilities, layout of rooms and domestic engineering facilities (e.g. electricity, gas and water supply, emission filtering, ventilation, air conditioning) to meet the requirements of the tests to be carried out. The disposal of all wastes should comply with the relevant safety provisions.

The allocation of space within the laboratory area should be such that contamination of the facility, equipment, staff and test systems is precluded, as is the undesired mixing of test substances and media. In particular, the cultivation and maintenance of test organisms should be separated from the preparation- and testing location.

For biological test methods, the corresponding statutory approval preconditions (e.g. requirements of animal protection legislation and provisions on the protection against epidemics) should also be met, in addition to the provisions related to laboratory and technical equipment.

15.3 Fixing the quality objectives

Quality objectives are fixed as the basis of decisions on the selection of the method and of the steps involved in quality assurance. Quality objectives cover both statistical parameters and statements on the selectivity or specificity of the test method.

15.4 Description of the test method

It is essential that all parts of the test method used are described as they are actually used, which equipment and instruments are used and which quality assurance measures are taken. Frequently occurring steps, which are a routine part of all tests or of a specific test method, can be laid down in standard descriptions [standard operating procedures (SOPs)], which means that it is not necessary to mention them specifically in each test plan. Typical of such procedures are the treatment of samples, the maintenance and calibration of measurement instruments, the cleaning of equipment and instruments, the breeding and cultivation of test organisms, safety and emergency facilities, etc.

All those involved in the test have to be informed of the scope, contents and time schedule of the test in advance. The work sheet has to contain clear information on the persons responsible, the objectives of the test, the carrying out of the test, the test methods used and the time and kind of measurements to be made.

15.5 Determination of test parameters

Prior to the practical application of a test method, the relevant test parameters (e.g. standard deviations and coefficients of variation) have to be determined. Validity criteria also need to be defined. In the case of standardized methods, statistical parameters and validity criteria are normally given in the standardized procedure.

Furthermore, the preparatory phase covers the selection of a suitable calibration strategy for the instruments, testing of suitability of the materials and examination of whether the method is adequate for the objective.

15.6 Quality control

15.6.1 Internal quality control

Internal quality control is an integral part of the overall test method. Normally, it is applied in those biological measurements which are conducted on a routine basis. It includes measures to identify, overcome and prevent errors, and should include the following steps:

— testing of actual preconditions in respect of staff, sampling, laboratory, equipment, test organisms, instruments and analytical methods;

— carrying out the calibration of instruments;

— carrying out tests with reference substances;

— keeping control charts;

— multiple determinations;

— plausibility control;

— validation.