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Water quality — Sampling —

Part 16: Guidance on biotesting of samples

Qualité de l'eau — Échantillonnage —

Partie 16: Lignes directrices pour les essais biologiques des échantillons

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by ISO/TC 147, *Water quality*, Subcommittee SC 6, *Sampling (general methods)*.

This second edition cancels and replaces the first edition (ISO 5667-16:1998), which has been technically revised.

A list of all parts in the ISO 5667 series can be found on the ISO website.

Introduction

Biological tests are suitable for determining the effect of environmental samples or chemical substances on the respective test organism under the specific standardized test conditions. Environmental samples are e.g. treated communal and industrial waste water, fresh water, aqueous extracts of solid material (e.g. leachates, eluates), pore water of sediments. The effect can be stimulative or inhibiting, and can be determined by the reaction of the test organism (e.g. death, growth, morphological and physiological changes or generally, changes in molecular mechanisms of action). Inhibiting effects can be triggered by toxic water constituents or by other noxious influences.

The toxicity measurable in the biological test is the result of the interaction between a single toxic substance, a mixture of substances or the constituents of an environmental sample and the test organism. The protective potential of the biological system, i.e. the test organism, for instance by metabolic detoxification and excretion, is an integral part of the biological test.

Apart from the direct toxic effect of one or more sample constituents, biological effects can be exerted by the combined action of all constituents of a sample. Such a combined effect includes the impact of, for example, substances which are not toxic *per se* but affect the chemical or physical properties of the test batches by interfering with the test specific additives (e.g. nutrients, salts) and, consequently, the living conditions for the test organisms. This applies for instance to oxygen-depleting substances, coloured substances or turbid matter which reduce light exposure.

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

The results of the biological test refer primarily to the organism used in the test and the defined conditions stipulated for the test procedure. A harmful effect stated by means of standardized biological tests can justify concern that aquatic organisms and biocoenosis 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 suborganismic tests, as important properties and physiological functions of intact organisms (e.g. protective integuments, repair mechanisms) are removed or deactivated.

In principle there is no test organism which can be used to test all the effects on the biocoenosis or the ecosystem possible under the various combinations 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 some issues should be taken into account during sampling and sample treatment in order to avoid a change in the sample properties. This applies to the method of sampling, including the sampling equipment and sample container as well as the transport to the laboratory. The method of sample pre-treatment and storage, as well as the preparation of, for example, stock solutions, may have an influence on the test result as well.

Furthermore, the sample to be tested can pose experimental problems on biotesting. Environmental samples (e.g. waste water, eluates) are complex mixtures and may contain, for example, sparingly soluble, volatile, unstable, coloured substances 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 of the test batch.

The statistical analysis of the data from biological testing of environmental samples should be conducted according to the current state of the art if not stipulated by the specific biotest standard.

Finally, it is recommended to implement and maintain a quality management system regardless if a laboratory is involved in testing of substances or environmental samples.

This document is one of a group of International Standards dealing with the sampling of waters and sediments and is intended to be read in conjunction with the other parts of the ISO 5667 series, in particular with ISO 5667-1, ISO 5667-3 and ISO 5667-15.

Water quality — Sampling —

Part 16: Guidance on biotesting of samples

1 Scope

This document gives practical guidance on sampling, pre-treatment, performance and evaluation of environmental samples in the context of performing biological tests. Information is given on how to cope with the problems of biotesting arising from the 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, for example, waters.

Primarily dealt with are substance-related problems concerning sampling and pre-treatment of environmental samples (e.g. waste water samples) for the performance of biotests.

This guidance is on ecotoxicological testing with organisms (single-species biotests; *in vivo* and *in vitro*). Some features addressed in this document also apply to biotests using single-cell systems (*in vitro* bioassays) and biodegradation studies as far as sampling and sample preparations are concerned. Testing of substances in the water solubility range is also addressed.

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

This document is applicable to biological tests for determining the effect of environmental samples like treated communal and industrial waste water, groundwater, fresh water, aqueous extracts (e.g. leachates, eluates), pore water of sediments and whole sediments. This document is also applicable to chemical substances.

This document is not applicable to bacteriological examination of water. Appropriate methods for bacteriological examination are described in other documents (see ISO 19458[17]).

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
blank
mixture of water and nutrients without test organism

3.2
cell density
x
number of cells per unit volume of medium

Note 1 to entry: Cell density is expressed in cells per millilitre.

[SOURCE: ISO 10253:2016, 3.1][6]

3.3
control
control medium (3.4), or *control sediment* (3.5), including organisms used in the test, without test sample

3.4
control medium
combination of dilution water and/or nutrient medium used in the test

[SOURCE: ISO 20079:2005, 3.6][18]

3.5
control sediment
defined artificial or natural sediment used in the test

3.6
dilution level
D
reciprocal value of the volume fraction of test sample in *dilution water* (3.7) in which the test is conducted

EXAMPLE 250 ml of waste water in a total volume of 1 000 ml (volume fraction of 25 %) represents dilution level $D = 4$.

[SOURCE: ISO 15088:2007, 3.2, modified — "waste water" replaced by "test sample"][13]

3.7
dilution water
water added to the test sample to prepare a series of defined dilutions

Note 1 to entry: The composition of the water is specified in the respective standard.

[SOURCE: ISO 20079:2005, 3.7, modified — "Note 1 to entry" has been added][18]

3.8
effective concentration
EC_x
concentration of the test material in water or sediment that causes *x* % change in response during a specified time interval

[SOURCE: ISO/TS 20281:2006, 3.8.1, modified — "quantal" has been removed from the term and abbreviated term; "soil" and "(e.g. immobility)" have been removed from the definition; the EXAMPLE and Notes 1 and 2 to entry are not included][20]

3.9
field blank
container prepared in the laboratory using reagent water or other blank matrix and sent with the sampling personnel for exposure to the sampling environment to verify possible contamination during sampling

[SOURCE: ISO 11074:2015, 4.5.3][9]

3.10
growth rate
proportional rate of increase in biomass per unit of time: (1/day)

[SOURCE: ISO 10253:2016, 3.2, modified — "specific grow rate" replaced by "growth rate"; formula and Note 1 to entry not included][6]

3.11
lowest ineffective dilution
dilution factor
LID
lowest ineffective dilution tested, expressed as *dilution level D* (3.6), at which no inhibition, or only effects not exceeding the test-specific variability, are observed

[SOURCE: ISO 15088:2007, 3.5][13]

3.12
nutrient medium
solution of nutrients and micronutrients in water which are essential for the growth of the test organism

[SOURCE: ISO 20079:2005, 3.17, modified — "duckweed" replaced by "the test organism"][18]

3.13
positive control
well-characterized *reference substance* (3.14) that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

[SOURCE: ISO 10993-12:2010, 3.14, modified — "any" removed before "well-characterized"; "material and/or substance, which" replaced by "reference substance that"][8]

3.14
reference substance
known substance to verify the sensitivity of the method

3.15
reference batch
mixture of dilution water, test specific additives and reference substance, including test organisms

3.16
replicate
one of a selected number of identical *test batches* (3.24) or identical *reference batches* (3.15)

3.17
sample
portion of material selected from a large quantity of material

Note 1 to entry: The method of sample selection can be described in the sampling plan.

Note 2 to entry: The material is from the environment (e.g. waste water, sediment or an eluate), a chemical substance or preparation or related material.

[SOURCE: ISO 16133:2004, 2.11, modified — Notes 1 and 2 to entry have been added][15]

3.18
sample pre-treatment
collective noun for all procedures used for conditioning a sample to a defined state which allows subsequent examination

Note 1 to entry: Depending on the requirements of the method sample, pre-treatment includes for example preservation and storage, centrifugation, filtration, homogenization, preconcentration and pH adjustment.

3.19
sample storage
process, and the result, of keeping a sample available under predefined conditions for a (usually) specified time interval between collection and further treatment of a sample

Note 1 to entry: Specified time is the maximum time interval.

[SOURCE: ISO 5667-3:2012, 3.3][2]

3.20
stock culture
culture of a single species to conserve the original defined species in the laboratory

[SOURCE: ISO 20079:2005, 3.21, modified — deleted "duckweed", "*Lemna*" "and to provide inoculum for the pre-culture"][18]

3.21
stock solution
solution with accurately known analyte concentration(s), prepared from chemicals with an appropriate purity

[SOURCE: ISO 11885:2007, 3.23][10]

3.22
storage time
period of time between filling of the sample container and further treatment of the sample in the laboratory, if stored under predefined conditions

Note 1 to entry: Sampling finishes as soon as the sample container has been filled with the sample. Storage time ends when the sample is taken by the analyst to start sample preparation prior to analysis.

[SOURCE: ISO 5667-3:2012, 3.4, modified — Note 2 to entry not included][2]

3.23
sub-sample
representative portion removed from a sample

[SOURCE: ISO 5667-19:2004, 3.7][5]

3.24
test batch
test medium including organisms used for testing

[SOURCE: ISO 20079:2005, 3.22][18]

3.25
test medium
mixture of test sample or test substance, dilution water and nutrients (without test organisms)

[SOURCE: ISO 20079:2005, 3.23, modified "combination" replaced by "mixture", after test sample added "or test substance", deleted "/or", "nutrient medium used in the test" replaced by "nutrients (without test organisms)"][18]

3.26
test sample
sample to be tested, after finishing all preparations

EXAMPLE Preparations include centrifugation, filtration, homogenization, pH adjustment and measurement of conductivity.

[SOURCE: ISO 13829:2000, 3.7][11]

3.27
test substance
chemical substance under investigation added to the test system

[SOURCE: ISO 15473:2002, 3.10][14]

3.28
test material
material to be tested

[SOURCE: ISO 17126:2005, 3.3, modified — EXAMPLES removed][16]

4 General guidance regarding test design

4.1 General

For each test, several replicates of the control and the treatment groups should be examined. The minimum number of replicates is usually prescribed in the respective standard. An example of how the required number of observations (replicates) can be calculated is given in 4.2.3.

It is recommended to minimize the influence of differences in testing conditions (e.g. light, temperature) for example by randomizing the design for location of the test vessels in the test chamber.

To assure that the laboratory test conditions (including the condition and sensitivity of the test organisms) are adequate and have not changed significantly, a reference substance should be tested as a positive control.

4.2 Replicates

4.2.1 General

Mainly three statistical approaches are performed in statistical analysis of the results from ecotoxicity tests with environmental samples.

a) determining the lowest ineffective dilution (LID) in testing of, for example, waste water;

b) two sample comparisons between the control (or reference batch) and either test or positive control batches (toxic standard);

c) computing point estimates [e.g. EC_{20}, LC_{50}(Lethal concentration)] from modelled concentration or dilution response relationships.

In ecotoxicity testing of environmental samples, the determination of a NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) is normally not intended and thus is not considered in this document. If the assessment of the NOEC should be exceptionally performed, consider ISO/TS 20281[20].

The number of replicates is mainly critical in hypothesis testing (e.g. two-sample comparisons) and is dependent on the variability of the respective endpoint to be evaluated, the minimum effect size which has to be detected by a statistical test and the statistical power. The estimation of an EC_x puts different demands on the study design than on the two-sample comparison (see ISO/TS 20281[20]).

4.2.2 Lowest ineffective dilution (LID)

In toxicity testing of, for example, waste water or eluates, the sample is diluted according to a defined scheme of dilutions (D). The lowest ineffective dilution (LID) denotes the most concentrated test batch at which no inhibition or mortality, or only effects not exceeding the test-specific limit occur (e.g. ISO 20079[18]: 10 % inhibition of the growth rate of *Lemna* spec.). D is expressed as the reciprocal value of the volume fraction of e.g. waste water in the test batch.

This approach does not require further statistical analysis (e.g. hypothesis testing).

4.2.3 Hypothesis testing — two-sample comparisons

Statistical two-sample comparisons play an important role in testing of environmental samples. They are performed in order to compare samples from various sample sites with a reference site (e.g. samples from a polluted area of running waters with a sample from an unpolluted reference site upstream) or to compare samples taken at different periods.

Under some circumstances, for example when it is only desired to ascertain whether a given dilution level exhibits an effect, a two-sample comparison involving a comparison of responses in a control and one test concentration or a positive control may be undertaken (see 10.2.5).

When performing two-sample comparisons, the type of endpoint data, i.e. whether quantal (qualitative) or metric (quantitative), variables under investigation is of decisive importance for the selection of the test procedure and calculation of the necessary sample sizes (replicates). In addition, a statistical test based on an analysis of variance (ANOVA) requires normal distribution and variance homogeneity of data.

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 for example, body length or biomass, metabolic rates, oxygen production, consumption rates or enzymatic transformation rates. The number of young animals produced may also be regarded as an approximate metric variable.

Formulae to calculate the required number of observations (replicates) are provided by statistical textbooks and papers (e.g. Reference [24]). As an example, to compare a metric variable (e.g. biomass) measured in a water or sediment sample from a polluted site and a reference site, a two-sample t-test is performed. The calculation of the required sample size is given in Formula (1) (see Reference [24]):

$$n \geq 2\left(\frac{\sigma}{\delta}\right)^2 \left(t_{\alpha,df} + t_{2\beta,df}\right)^2 \quad (1)$$

where

n is the number of replicates;

σ is the true standard deviation of the end point;

δ is the smallest true difference;

df are the degrees of freedom (here: $df = n_1 + n_2 - 2$);

α is the desired significance level (e.g. 0,05);

β is the desired type II error [$1 - \beta$ is called “power” of the statistical test and denotes the desired probability that a difference will be found to be significant (if it as small as δ)];

$t_\alpha \mid t_{2\beta}$ are the values from a two-tailed t table with df degrees of freedom.

It is necessary to know only the ratio of σ to δ, not their actual values (e.g. given the coefficient of variation is 20 % and the desired detectable difference 10 % the ratio is two). Consider statistical textbooks or ISO/TS 20281[20] for formulae of other test procedures.

4.2.4 Concentration and dilution response relationship

Concentration and dilution response modelling can be used to determine definite effect sizes (e.g. EC_{10}, EC_{50}, LC_{50}) evoked by the volume fractions of the test sample. It should be used to analyse the effects of reference substances in order to demonstrate the performance of the test system (e.g. ISO 20079[18]).

The test is seen as valid if the obtained EC_{50} for the reference substance is bracketed by a lower and upper limit EC_{50}, determined in previous ring tests.

The primary demand on the design is to have a sufficient number of concentration (dilution) groups. This might be at the expense of the number of replicates per group (e.g. keeping the total size of the experiment the same), since the precision of the estimated EC_x depends more on the total size of the experiment rather than on the sample size per concentration or dilution group.

Again, the type of endpoint data, i.e. whether quantal (qualitative) or metric (quantitative) variables are determined, is of decisive importance for the test design and statistical method. For more information, see ISO/TS 20281[20].

5 Evaluation

5.1 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 measures of dispersion (descriptive statistics).

In many cases, this is followed by more extensive statistical processing which aims to determine concentration or dilution 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 critical examination of the data.

5.2 Statistical analysis

The statistical analysis of the data from ecotoxicity testing of environmental samples should be conducted according to the current state of the art as stated in ISO/TS 20281[20] or the respective standard of the ecotoxicity test. In particular, the following should be considered.

— The data should be completely documented in tabular or graphical form.

— An appropriate statistical method should be chosen based on the type of data scale.

— With hypothesis testing, a measure of statistical power of the conducted statistical test procedure should be provided, e.g. the minimum detectable (= significant) difference (see References [24] and [25]).

— With concentration or dilution response modelling, confidence limits (e.g. 95 % confidence limits) should be provided for the reported EC_x or LC_x).

— It is highly recommended to perform all statistical calculations using validated statistical software.

6 Sampling and transportation

6.1 General

Sampling is the first step in carrying out biological, chemical and physical examinations. The goal of sampling should be to obtain a representative sample for the research question assessed and to supply it to the laboratory in the correct manner.

Environmental samples are susceptible to change as a result of physical, chemical or biological reactions which can take place between the time of sampling and the analysis. If the necessary precautions are not taken during sampling, transportation and storage, the nature and rate of these reactions are often such that the sample can be changed substantially and will no longer be representative of the original sample. The extent of these changes is dependent on the chemical and biological nature of the sample,

the material of the sampling equipment (sampling vessel and sample container), the transportation and pre-treatment (e.g. storage, preservation). Errors caused by improper sampling and sample pre-treatment cannot be corrected.

This document is intended to be used in conjunction with ISO 5667-1[1] which sets out the general principles and provides guidance on the design of sampling programmes and sampling techniques for all aspects of sampling of water (including waste water, sludge, effluents and bottom deposits), e.g. the choice of representative sampling points, time and frequency of sampling, sampling techniques, sample equipment for physical or chemical characteristics, avoidance of contamination, transportation to and storage of samples at the laboratory, sample identification and records.

For the determination of physico-chemical parameters ISO 5667-3[2] gives guidance when spot or composite samples cannot be analysed on site and have to be transported to a laboratory for analysis. It establishes general requirements for handling and preservation of samples, sample transportation, sample reception and identification as well as sample storage. For the preservation and handling of sludge and sediment, see ISO 5667-15[4].

In general, the sampling approach for performing biotests is compatible with the one for chemical analysis. In any case, consultation with the laboratory conducting the biotest is strongly recommended.

6.2 Sampling equipment

6.2.1 General

The provisions on sampling equipment, especially on the material used, apply to both the sampling vessel as well as to sample containers. The type of container and sample equipment should be chosen in agreement with the testing laboratory since for some biotests only certain materials are suitable for sampling, transportation and storage. Specific requirements for the sampling material described in a given national or international biotest standard are mandatory.

6.2.2 Sample container

The choice of sample container is of major importance and ISO 5667-1[1] and ISO 5667-3[2] provide guidance on this subject.

The majority of the following instructions and recommendations are cited from ISO 5667-1[1].

The sample container should be designed to preserve the composition of the sample from losses due to adsorption and volatilization, or from contamination by foreign substances. The most frequently encountered problems consist of adsorption of chemical substances onto the walls of the sampling vessel or sample container, contamination prior to sampling caused by improper cleaning of the sampling vessel or sample container, and contamination of the sample by the material constituting the sampling vessel or sample container.

The sample container used to collect and store the sample should be chosen after considering, for example, resistance to temperature extremes, resistance to breakage, ease of good sealing and reopening, size, shape, mass, availability, cost, potential for cleaning and re-use.

For light-sensitive materials, light-absorbent glass should be used. Stainless steel should be used for taking samples of water under high temperature and/or pressure, or when sampling for trace concentrations of organic material.

In addition to the desired physical characteristics described above, the sample containers used to collect and store the samples should be selected by taking into account the following predominant criteria (especially when the constituents to be analysed are present in trace quantities):

a) minimization of contamination of the sample by the material of which the container or its stopper is made, for example, leaching of inorganic constituents from glass (especially soft glass) and organic compounds and metals from plastics and elastomers (plasticized vinyl cap liners, polychloroprene jackets);

b) ability to clean and treat the walls of the containers, to reduce surface contamination by trace constituents such as heavy metals;

c) chemical and biological inertness of the material of which the container is made, in order to prevent or minimize reaction between constituents of the sample and the container;

d) sample containers which can also cause errors by adsorption of chemical determinants. Trace metals are particularly liable to this effect, but other determinants (e.g. detergents, pesticides and phosphate) can also be subject to error.

The sample container should be resistant to heating and freezing and it should be autoclavable and easy to clean. Polypropylene (PP), polytetrafluoroethylene (PTFE) or polyethylene (PE) containers are appropriate, but polyethylene is not autoclavable. Glass bottles are generally (but not always) suitable for organic chemical compounds and biological species.

The volume, shape and material of the sample containers are dependent on the nature of the sample, the number of replicates, the volume required for the biotests and the necessity of preserving and storing the samples prior to further processing.

The volume of sample collected should be sufficient for the required analyses and for any repeat analyses. The use of very small sample volumes can cause the samples collected to be unrepresentative. In addition, small samples can also increase problems of adsorption because of the relatively high area to volume ratio.

6.3 Filling status of sample containers

To minimize possible impacts on the sample during transportation it is recommended to fill the containers completely.

When freezing is envisaged for preservation, sample containers should be filled only up to an extent which allows expansion of volume (prevention of breakage).

Problems related to partial filling can include:

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

— interaction with gas phase, leading to stripping, and

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

If completely filled sample containers are transported as recommended, the volume reduction for freezing takes place in the laboratory after homogenization.

6.4 Sample identification and records

Sample containers should be clearly and unambiguously marked, so that subsequent analytical results can be properly related.

A unique identifier, with at least sample number, sample date and location should be on the label of the sample container. All other information is additional and depends on the objectives of the particular measurement programme and requirements of the successive biotest and can be noted in the sample report. Container labels should withstand wetting, drying and freezing without detaching or becoming illegible. The labelling system should be waterproof to allow use on site.

Labels or forms should always be completed at the time of sample collection.

6.5 Sub-sampling

Sub-sampling can be required for a couple of reasons. Frequently sub-sampling is necessary for example to get a retained sample or if different biotests cannot be performed in parallel or if different tests require the sample to be handled and kept in a particular way.

Sub-sampling can be realized soon after sampling on site, in the laboratory before further treatment or after thawing. The time is dependent on the research question.

The process of sub-sampling should ensure that the sub-samples remain representative. For sub-sampling the sample should be thoroughly mixed before distribution. To obtain sub-samples of equal quality, it should be ensured that the bulk sample maintains homogeneity during the sub-sampling process (e.g. by continuous shaking or stirring). This holds particularly in the case of two-phase mixtures, e.g. waters containing suspended particles.

Remaining sub-samples stored frozen separately should be saved until the final evaluation has been made.

6.6 Transportation

Samples should be delivered to the laboratory as soon as possible after sampling.

Keep the sample container frost- and break-proof during transportation, protected from exposure to light, temperature increases and external contamination.

Cooling or freezing (see 7.2) procedures should be applied to the samples in order to increase the time period available for transport and storage. Cooling should commence as soon as possible after sampling for instance in cool boxes with ice (no dry ice), frozen gel packs or cooling elements. A cooling device in the transport vehicle is also suitable. A cooling temperature during transport of 2 °C to 8 °C has been found suitable for many applications. The suggested cooling temperature applies to the surrounding of the sample (e.g. inside the cooling box) and not for the sample itself.

Cooling and freezing procedures applied should be in line with instructions from the analytical laboratory.

If samples are preserved by cooling, the time span between sampling and analysis (storage time) should remain within the time set for the respective biotest. Usually this storage time is 48 h.

For further information see ISO 5667-3[2], ISO 5667-14[3] and ISO 5667-15[4].

6.7 Contamination during sampling

Avoiding contamination during sampling is essential. All possible sources of contamination should be taken into account and the appropriate control applied if necessary. Potential sources of contamination include the residue of earlier samples remaining on sample containers, funnels, scoops, spatulas and other equipment, contamination from the sampling site during sampling or contamination of bottle caps or tops by dust or water.

For detailed information, see ISO 5667-14[3].

6.8 Sampling quality control techniques

ISO 5667-14[3], amongst others, gives guidance on sampling quality control techniques. Such techniques are in particular:

— **Field blank samples**: This technique can be used to identify any errors relating to contamination of sample containers and the sampling process. Field blank samples are blank samples which are taken from the laboratory into the field, treated as samples and analysed as a check on sampling procedures.

— **Filtration recovery**: This technique can be used to identify any errors relating to contamination of sample containers and the sampling process associated with filtration of samples.

When there is a requirement to filter samples on site or in the laboratory, then field blanks and/or standard quality assurance samples should be processed using the same filtering procedures as for real samples.

7 Pre-treatment

7.1 General

Pre-treatment comprises the treatment of a sample to a homogeneous test sample under laboratory conditions for subsequent examination.

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

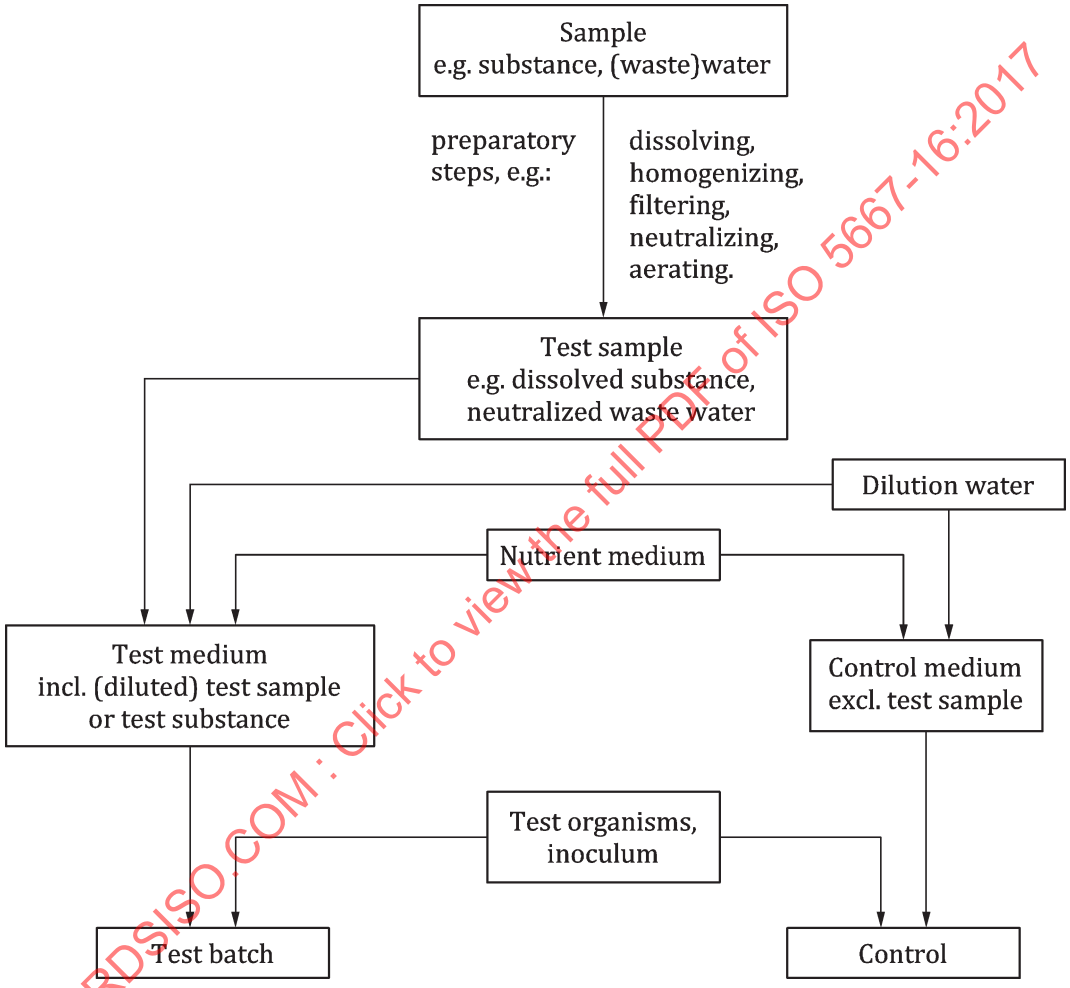

Figure 1 — Preparation of samples for biotesting

7.2 Preservation and storage

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.

Cool down samples to temperatures between 2 °C and 8 °C if testing almost immediately after sampling is not possible. The samples should be kept in the dark to prevent algal growth. When cooled to this range and stored in the dark, most samples are normally stable for up to 48 h.

Freeze down water samples to ≤–18 °C as soon as possible after sampling if it is not possible to start performance of the test within 48 h. The time required for freezing and thawing should be minimized by reducing the sample volume, i.e. the size of the sample container. In general, it is appropriate to use one-litre containers for freezing (filled with max. 0,5 l to 0,7 l of sample). For tests requiring larger volumes, the sample should be homogenized and split into sub-samples (see 6.5).

A storage period of up to two months is best practice as maximum storage period for most biotests.

The use of biocidal preservatives is excluded for the purpose of biotesting. The addition of highly concentrated acids or bases to stabilize the samples, e.g. HCI or NaOH, is not recommended either. Experience has shown that the quality of waste water can be affected during both freezing and thawing. Specific requirements for sample preservation and storage described in a given international or national biotest standard are mandatory. If required, the storage method and period should be determined for each sample type and biological test method separately.

Freezing especially requires detailed control of the freezing and thawing process in order to return the sample to its initial equilibrium after thawing.

The recommended way of preserving sediment samples is to cool them down to temperatures between 1 °C and 5 °C and store them in the dark (see ISO 5667-15[4]).

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

7.3 Thawing

Samples stored frozen are thawed on the day of testing shortly before use. A warm water bath at a temperature not exceeding 25 °C, together with gentle shaking, are recommended to avoid local overheating. Alternatively, the sample can be thawed in the dark at a temperature between 2 °C and 8 °C overnight and used directly for the test.

Complete thawing of a sample before use is essential, as the freezing process can lead to the concentration of certain components in the inner part of the sample which freezes last. Do not use microwave treatment for thawing the sample. For the performance of biotests, a once thawed sample should not be frozen again or stored in a fridge for testing on subsequent days.

7.4 Homogenization

An even distribution of all soluble and particulate components should be ensured (e.g. by gentle agitation or vigorous shaking). During this treatment step, attention should be given to the potential loss of volatile ingredients. Ultrasonic treatment or high-speed mechanical dispersion (e.g. homogenizer) are not applied for environmental samples because the sample can be changed by these methods substantially, especially in the presence of particulate matter. For poorly soluble substances, see 10.2.

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

7.5 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 in aquatic test systems with the requirements of test organisms (e.g. clogging of fish gills, impairment of filter feeding of daphnids and light limitation of algae).

High background particle numbers can disturb the measurements (e.g. when using a particle counter or a spectrophotometer). Microscopic counting is strongly impaired as well. Continuous dosing is rendered unreliable by clogging and blockage of tubing.

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

Filtration, centrifugation and other separation methods, however, involve the risk that active components, bound to the particles, are removed prior to the test. Moreover, problems related to filtration (e.g. adsorption on and leaching of filter material) need to be taken into account. Sedimentation

and centrifugation circumvent these problems. Centrifugation (e.g. 10 min at 4 500 *g* ± 1 500 *g*) is, in general, preferable to filtration.

When carrying out aquatic 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. The required quantity of supernatant can be sampled using a pipette. The pipette tip should be positioned in the centre of the container and half way between the surface of the deposited fraction and the surface of the liquid. Another method of separation of gross particles is a coarse filtration (>50 µm). The separated particle mass can be examined separately.

The filter material should be made of inert material and the filters should be rinsed with high-purity water prior to use to reduce the risk of contamination of the test material with toxic residues. Check filters for toxic residues by filtration of dilution water or medium with subsequent testing in the biotest. Adsorption of the test material can be reduced by preconditioning filters with the respective solutions of the test material. Filtration can be carried out under pressure or with vacuum.

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

Water samples 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.

For sample preconcentration procedures (see 7.6), filtration of the samples is mandatory, as particles might block the cartridge and prevent a successful extraction. For this purpose, glass fibre filters with a pore size of around 1 µm are suitable.

Any method used for separation of particles should be reported in the test report.

7.6 Preconcentration

7.6.1 General

For certain procedures (e.g. the determination of cause-effect relationships for a risk assessment of environmental contamination or benchmarking of samples), the testing of preconcentrated samples or fractions thereof with biotests in combination with chemical analysis is a common procedure.

A sample preconcentration might also be appropriate if only very low concentrations of pollutants are present in the environmental sample or if some specific *in vitro* biotests should be used. Particularly, an enrichment of water samples allows the detection of substances in low concentrations by means of short-term *in vitro* biotests.

Preconcentration of samples might increase the concentration not only of harmful substances but of other water constituents as well. These constituents might interfere with the biotest in higher concentrations.

Preconcentration typically involves a procedure where a range of compounds is selectively enriched from a sample (i.e. an extraction). In this procedure, matrix components such as salts (e.g. nutrients, which might mask the toxicity of the samples) as well as other potentially toxic compounds such as metals (see Reference [27]) may be removed from the sample. It is essential to take into consideration that preconcentration is selective. The extent of this selectivity depends on the procedure applied.

It should be kept in mind that, to date, it is not possible to extrapolate from effects measured in acute tests with preconcentrated samples to chronic effects of the original sample. An extrapolation would require a thorough calibration of the extraction method and a short-term biotest indicating chronic toxicity. An appropriate example for such an extrapolation has so far not been reported.

If a preconcentration procedure is applied (e.g. if there is no sensitive method available to test the original sample) results from a preconcentrated sample should be interpreted with caution. The results are more difficult to relate to the original sample depending on 1) the degree of selectivity of the preconcentration that has occurred and 2) the concentration factor applied.

7.6.2 Extraction methods

During preconcentration, the original composition pattern of water ingredients is altered, for example:

— liquid/liquid extraction with organic solvents and solid phase extraction by adsorption on solids (e.g. C18-cartridge) are particularly efficient for organic contaminants. Ionic strength and osmotic pressure of the sample can be lowered. Toxic ions, and other water ingredients possibly contributing to the effect (e.g. masking it), such as humic acids, can be excluded;

— evaporation and freeze-drying and sorbent-extraction can lead to a loss of volatile substances; evaporation and freeze-drying also enhance the ionic strength and osmotic pressure;

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

If the concentration of a specific compound is increased above its solubility, precipitation or flocculation of previously dissolved substances can occur.

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

Such issues should be assessed thoroughly before an extraction procedure is selected. For more information on this topic, see References [27] and [28].

7.7 pH adjustment

The selection of the pH value to which the sample is to be adjusted is governed by the objective of the test and the physiological requirements of the test organism.

In many biotests, samples with a pH value ≥6 and ≤9 can be tested without adjustment. pH values between 6 and 9 (which are usually tolerable for aquatic biota) will permit the expression of ionizable toxicants that would otherwise be masked by pH conditions outside this range.

Samples with extreme pH values exceeding the tolerance limits of most test organisms (usually pH <6 or >9, depending on the organism) should be adjusted to pH 7,0 ± 0,2. The physiological limits of the organisms should be defined in the respective standards. Exceeding the neutral pH value should be avoided.

The concentration of the acid or base required for adjustment should be such that the volume change is as small as possible.

The addition of acid or base should not lead to precipitation or complexation. The bioavailability of substances is reduced when they are removed from the test media. The addition of acid or base should not influence the test organism unduly. Usually, hydrochloric acid or sodium hydroxide solutions are recommended.

Adjustment 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.

8 Apparatus and equipment

8.1 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, polytetrafluoroethylene (PTFE), stainless steel. Tubing connections should be as short as possible and replaced from time to time. New tubing should be rinsed several times before use, in order to minimize leaching of plasticizers or other foreign matter from the material. 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.

8.2 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, solvents (ethanol, acetone, methanol), 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.

9 Impairment of test performance

9.1 Problems and preventive measures for samples containing removable ingredients

9.1.1 General

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

— evaporation of volatile substances;

— foaming of surface-active agents;

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

— precipitation;

— flocculation;

— biodegradation;

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

— weak partition or binding in case of liquid-liquid or sorbent extraction.

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

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. Furthermore, the ratio of test organism or cells to the exposure volume determines the potential loss of compounds from the sample, particularly for lipophilic compounds. 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.

9.1.2 Volatilization

Particularly in test methods conducted in open systems or 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 (for further information see ISO 14442[12]). 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.

Volatile substances not only strip from the test system but also from one test batch to another and higher concentrated test batches can influence lower concentrated. This should be considered when incubating the test batches in an incubator or when working with microplates.

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

9.1.3 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 sample and should generally be avoided except in special cases (e.g. biodegradation studies).

9.1.4 Adsorption

Hydrophobic substances can be adsorbed to vessel walls and are no longer fully bioavailable, especially at low concentrations. In order to prevent large losses of substance, vessels and material for pipetting and transferring may be pre-incubated with the sample at the envisaged concentration prior to the test. After pre-incubation the sample should be discarded and replaced by a fresh sample in order to prepare the test batch.

9.1.5 Precipitation/flocculation

Water, waste water and organic/inorganic solids/liquids can contain components that modify the composition of the test batches (by precipitation of a limiting nutrient, complexation of essential elements, addition of nutrients), and subsequently can cause effects on the test organism (e.g. algal growth) not related to toxic components. For further guidance, see ISO 14442[12].

9.1.6 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. In dependency on test duration, for example, the transformation of an environmental sample is more or less complex and an accepted element of biological tests. Usually, it is impossible to prevent biodegradation in static test systems.

Some ingredients (e.g. isocyanates, esters and anhydrides) hydrolyse in water. This 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.

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

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

In some aquatic 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 end point 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 to 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).

10 Preparation of stock solutions and test batches

10.1 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 sample.

10.2 Poorly soluble substances

10.2.1 General

The Organisation for Economic Co-operation (OECD)[22] has developed guidance for the aquatic toxicity testing and assessment of difficult substances and mixtures. 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.

10.2.2 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 l of distilled water or preferably in test medium (see ISO 14442[12]), e.g. for 24 h to 48 h, in the dark at the intended test temperature, adjusted to the solubility and stability of the test compound. Note that the OECD[22] recommends that the maximum concentration for test medium preparation by direct addition should be below 50 % of the water solubility. ISO 14442[12] recommends that for preparing a saturated solution, the minimum quantity necessary should be used in order to avoid the enrichment of impurities with higher solubility. The weighed portion for preparing the test concentration should be indicated. Following phase separation, the undissolved phase is fully separated by filtration (where necessary using a membrane filter, pore size 0,2 µm to 0,45 µm) or preferably by centrifuging. The dilution series is prepared with the aqueous phase.

There is a variety of mechanical and chemical means to reach the saturation concentration, such as sonication/high speed grinder, temperature increase, pH adjustment, use of volatile, non-aqueous miscible solvents which evaporate after dosing or dissolution of the substance in a water-miscible, nontoxic solvent, sorption of the substance on to an inert carrier. It should be noted, 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. According to Reference [22], the use of solvents should be restricted to situations where no other acceptable method of media preparation is available because of the potential for interaction with the test substance resulting in an altered response in the test. If solvents are used, their effects on the test results need to be determined by appropriate controls (solvent or carrier controls) to be included in the test design. The maximum solvent volume should be limited to <0,1 ml/l. The OECD[22] and ISO 14442[12] describe some principles to be considered when solvents are used.

In degradation studies, poorly soluble substances may also be tested above the solubility limit by direct dosage of an appropriate weight or volume. The use of inert carriers such as polyethylene films or microscopic slides or the application of undegradable solvents (e.g. dimethyl sulfoxide) can support a higher distribution rate for undissolved matter and a larger contact area between the microorganisms and the substance. A number of suitable biodegradation tests are described in ISO/TR 15462[21].

10.2.3 Dispersions and emulsions

The OECD[22] does not recommend testing of aqueous dispersions and emulsions for the following reasons:

— The effects observed in toxicity tests are generally best explained when considered in relation to exposure concentrations of dissolved test substance;

— The presence of non-dissolved material presents significant difficulties for the determination of exposure concentrations;

— Non-dissolved material present in test media has the potential to exert physical effects on test organisms which are unrelated to toxicity.

However, testing of emulsions might be performed where there is a regulatory requirement, such as assessing oil dispersing agents or testing of formulated products. Also, test substances which have an inherent tendency to form an aqueous dispersion or emulsion, such as surfactants and detergents, might be tested in emulsions.

Stable dispersions or emulsions can sometimes be produced by the simple expedient of physically mixing the test substance with the aqueous phase. The use of chemical dispersants or emulsifying agents is not generally advocated because of the potential for physical-chemical interactions influencing the apparent toxicity of the test substance. ISO 14442[12] describes some practical advice when dispersing or emulsifying agents are used.

10.2.4 Special problems with mixtures of substances or technical products

Mixtures comprising a complex mix of individual substances with different solubility and physical-chemical properties are referred to as “complex mixtures”. The OECD[22] describes different approaches to media preparation and/or testing of multicomponent substances. The toxicity of complex multi-component substances, which are only partially soluble in water, can be determined by preparing water-accommodated fractions (WAFs) containing only the fraction of multi-component substances that is dissolved and/or present as a stable dispersion or emulsion.

WAFs are prepared individually and not by serial dilution of a single stock WAF. The multi-component substances are added directly to water and mixed for a period of time sufficient to achieve an equilibrated concentration of dissolved and dispersed or emulsified components in the aqueous phase (nominal concentrations = loading rate). The required mixing and settling time (to allow phase separation) normally is determined in pre-tests. Generally, any non-dissolved test material which has sedimented or precipitated out in the test vessels should be removed from the test media using, for example, a separating funnel. When the WAF is filtered through suitable filters, a water-soluble fraction (WSF) is obtained (see ISO 14442[12]). The results for partially soluble mixtures are referred to as loading rates. The effect concentrations might also be referred to as measured concentrations of the test substance in the WAF.

10.2.5 Limit test

Under some circumstances, e.g. risk assessment or labelling purposes, information on the effect of defined concentration is sufficient. The limit test is a two-sample comparison involving a control and one test concentration. The limit test might be appropriate if:

— there is sufficient evidence (e.g. from the results of a preliminary toxicity test) that the test substance does not cause any significant adverse effects up to a concentration of 100 mg/l or up to its limit of water solubility (whichever is the lower). Then a limit test at 100 mg/l or at the limit of water solubility is generally considered to provide the appropriate information needed for risk assessment or classification and labelling purposes;

— a screening study is targeted to provide an answer exclusively to the question which, if any, effect occurs at a given concentration or dilution. For such investigation, there is no need to expose the test organisms to an extended range of concentrations or to a complete dilution series;

— test animal welfare considerations need to be balanced against the information gained from testing a full concentration range of a test substance versus a given limit concentration (the latter, depending on the specific issue, might be sufficient to draw the right conclusions).

Limit tests are not suitable for the detection of any specific effect which might be masked by acute toxicity in higher concentrations of the investigated compound or sample. In such cases, the dose response relationship is not monotonic.

11 Quality assurance for biotesting

11.1 General

For regulatory purposes, two quality management systems apply for biotesting. For the analysis of environmental samples (e.g. waste water, surface water, sediments and soils) ISO/IEC 17025[19] is an agreed quality management system for the laboratory. Authorities often require laboratories working in this sector to be accredited to this standard. For non-clinical testing of chemicals, pesticides and medicinal products, the OECD provisions on Good Laboratory Practice (GLP)[23] have been implemented in European law by Directive 2004/10/EC[29]. Laboratories that work under GLP have to be certified by authorities.

ISO/IEC 17025[19] includes two main sections: management requirements and technical requirements. Management requirements are related to the operation and effectiveness of the quality management system and refer to the assignment of responsibilities, the control of documents (approval, review) and the performance of internal quality audits. Technical requirements address the competence of staff, the calibration and validation of methods, the accuracy of the equipment and the clear, unambiguous and objective reporting of the test results. One aim of ISO/IEC 17025[19] is the permanent improvement of the quality through monitoring and control of any nonconformity, analysis of the causes and selection and implementation of corrective actions.

The aim of the GLP principles is to obtain high quality data of non-clinical health and environmental safety studies and to agree on mutual acceptance of these data for the evaluation of chemical products. The GLP principles describe the responsibilities of the test facility management, of the study director and of the quality assurance personnel. Each study is planned in detail in a study plan to be approved by the study director, the sponsor and the quality assurance. All records (e.g. study plan, raw data, final report, qualification and training) as well as samples and materials must be stored in archives for a defined time period.

11.2 Quality assurance in the context of the investigation of environmental samples

Laboratories that perform biotests with environmental samples for regulatory purposes such as waste water permits should have established a quality assurance system according to ISO/IEC 17025.[19] The GLP principles are not intended to be applied for environmental samples. If the implementation of ISO/IEC 17025[19] is not practicable (e.g. for research and development organizations such as