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GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURES

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GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURES

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About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 29 industrialised countries in North America, Europe and the Pacific, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised Committees and subsidiary groups composed of Member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's Workshops and other meetings. Committees and subsidiary groups are served by the OECD Secretariat, located in Paris, France, which is organised into Directorates and Divisions.

The work of the OECD related to chemical safety is carried out in the **Environmental Health and Safety Programme**. As part of its work on chemical testing, the OECD has issued several Council Decisions and Recommendations (the former legally binding on Member countries), as well as numerous Guidance Documents and technical reports. The best known of these publications, the **OECD Test Guidelines**, is a collection of methods used to assess the hazards of chemicals and chemical preparations. These methods cover tests for physical and chemical properties, effects on human health and wildlife, and accumulation and degradation in the environment. The OECD Test Guidelines are recognised world-wide as the standard reference tool for chemical testing.

More information about the Environmental Health and Safety Programme and its publications (including the Test Guidelines) is available on the OECD's World Wide Web site (see page 8).

The Environmental Health and Safety Programme co-operates closely with other international organisations. This document was produced within the framework of the Inter-Organisation Programme for the Sound Management of Chemicals (IOMC).

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, FAO, WHO, UNIDO and the OECD (the Participating Organisations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. UNITAR joined the IOMC in 1997 to become the seventh Participating Organisation. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

FOREWORD

Guidance for the aquatic toxicity testing and assessment of difficult substances and mixtures was identified as a high priority requirement by the National Co-ordinators of the Test Guidelines Programme (TGP) and the Risk Assessment Advisory Body (RAAB) of the OECD. The OECD Task Force on Ecotoxicology for priority setting in development and revision of Test Guidelines and Guidance documents appropriate for pesticides testing also gave high priority to the development of a guidance document for the testing of difficult substances.

The first draft proposal for an OECD guidance document on the aquatic toxicity testing of difficult substances and mixtures was prepared following a meeting of the Expert Panel on the 2nd and 3rd of April 1998 in Paris, France. It was circulated to National Experts for review in March 1999 through the National Co-ordinators of the Test Guidelines Programme. Comments were received from twelve countries (Australia, Belgium, France, Germany, Hungary, Italy, Japan, the Netherlands, Sweden, Switzerland, The United-Kingdom and the United States) and industry. A revised draft was circulated in January 2000 to National Co-ordinators for approval.

The revised proposal was well appreciated and the proposed guidance document was generally fully accepted. However, Denmark, Germany, the Netherlands, the United-Kingdom and the United-States made several additional comments. The document was again revised taking into account the amendments suggested and then submitted to National Co-ordinators of TGP for final approval. In May 2000, the 12th National Co-ordinators Meeting approved this last version and agreed that the draft Guidance Document be submitted to the Joint Meeting for declassification.

The Joint Meeting of the Chemicals Committee and the Working Party on chemicals, Pesticides and Biotechnology recommended that this document be made public. It is being published on the responsibility of the Secretary General of the OECD.

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Glossary of important terms used in the Guidance Document[1]

Substance [2]	Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substances or changing its composition.
Mixture [2]	Mixtures or solutions composed of two or more substances in which they do not react.
Preparation	Deliberate physical mixture of substances.
Multi-component substances [3]	Mixtures comprising a complex mix of individual substances with different solubilities and physical-chemical properties. In most cases, they can be characterised as a homologous series of substances with a certain range of carbon chain length/number or degree of substitution. These materials are frequently referred to as "complex mixtures". But, in this Guidance Document, these are referred to as "multi-component substances.
Water solubility	The maximum attainable concentration or concentration at thermodynamic equilibrium between aqueous phase and solid (or liquid or gaseous) pure phase. Concentration in pure water is meant.
Saturation concentration	The maximum dissolved concentration of a test substance that can be achieved under the test conditions.
Poorly (or sparingly) water-soluble substance	Substance with a limit of water solubility of <100 mg/l.
Water-miscible solvent	A solvent in which a test substance can be dissolved to facilitate preparation of stock solutions and/or treatment of test media.
Suspension	A stable dispersion of solid particles in a liquid
Emulsion	A stable dispersion of liquid droplets in a liquid. (Note: both liquids are no miscible)
Chemical dispersant / emulsifying agent	A chemical reagent (e.g. surfactant) which aids in the production of a suspension / emulsion of a substance
Critical micelle concentration	A maximum concentration of the freely solubilised surfactant in water.
Stable exposure concentration	A condition in which the exposure concentration remains within 80-120% of nominal over the entire exposure period.
Water-accommodated fraction (WAF)	An aqueous fraction containing the dissolved and/or suspended and/or emulsified fraction of a multi-component substances or a mixture.
Loading rate	The ratio of test material to water (in mg/l) used in the preparation of a WAF.

1. All terms and their descriptions should be considered as working definitions for the purpose of this Guidance Document only.
2. The definition is cited from a paper (ENV/JM/HCL(99)11), entitled "Step 2 Proposal for Harmonised Classification Criteria for Mixtures" and therefore considered as a provisional definition.
3. Consideration is given to the consistency with the definition of "multi-component substances" (or "complex substances") in Chapter 1 of a draft "Guidance Document on the Use of the Harmonized System for the Classification of Chemicals which are Hazardous for the Aquatic Environment" (ENV/JM/HCL(2000)11).

Introduction

Guidance for the aquatic toxicity testing and assessment of difficult substances and mixtures was identified as a high priority requirement by the National Co-ordinators of the Test Guidelines Programme and the Risk Assessment Advisory Body of the OECD. An Expert Panel was therefore established to review relevant literature and develop a guidance document.

The first draft guidance document was prepared following a meeting of the Expert Panel on the 2nd and 3rd of April 1998 in Paris, France (see Annex 5). The group considered relevant documents published by the United Kingdom Department of the Environment (DoE, 1996), the United States Environmental Protection Agency (US EPA, 1996a), the International Organization for Standardization (ISO, 1997), the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC, 1996), the Oil Companies European Organisation for Environment, Health and Safety (CONCAWE, 1992) and the Danish Water Quality Institute (VKI, 1998).

The members of the Expert Panel agreed that the document "Guidance on the Aquatic Toxicity Testing of Difficult Substances" published by the UK Department of the Environment should form the basis for the OECD guidance document. The text of the Guidance Document is therefore based, to a large extent, on the UK document but with amendments and additions as considered appropriate by the Expert Panel and consequent review by national experts.

The first draft was circulated for review in March 1999 to national experts through the OECD National Co-ordinators of the Test Guidelines Programme. A revised draft was submitted to National Co-ordinators for approval in January 2000. Additional comments were received, therefore a revised version was prepared and again submitted to National Co-ordinators for approval in April 2000. It was approved at the 12th National Co-ordinators Meeting in May 2000.

Scope

This document provides guidance for the testing of substances, and mixtures, classed as "difficult to test" for the purposes of determining their aquatic toxicity. Whilst the guidance given is targeted towards tests described by the OECD Guidelines identified in Table 1 it may also be relevant to other tests.

The guidance relates to the practical aspects of carrying out valid tests with "difficult" test substances and mixtures and presenting the results. As such it considers those substances and mixtures with the properties described in Table 2 that require modifications or additions to standard testing procedures. Guidance herein does not extend to interpretation of results, after testing of these substances, or their use in classification and risk assessment.

The guidance is considered to reflect good practice but it is important to recognise that some test substances and mixtures will present specific scientific and technical issues that may fall outside its scope. It is also important to acknowledge that the guidance may not be applicable or appropriate where the test results are to be used for applications which fall outside the scope of the guidelines. Under such circumstances it is important to consult with the relevant end user of the data to ensure that appropriate testing procedures are agreed upon and followed.

This document is divided into five substantive sections covering:

- guidance for carrying out a preliminary assessment of test substance stability;
- general considerations on selection of exposure regimes;
- development of media preparation and exposure systems;
- sampling of test media for analysis, and
- calculating and reporting test results.

Specific guidance on testing of metals and metal compounds can be found in a draft report of an OECD Workshop on Aquatic Toxicity Testing of Sparingly Soluble Metals, Inorganic Metal Compounds and Minerals (OECD, 1995). Where appropriate, the recommendations made at the workshop have been incorporated into this document.

It should also be mentioned that revision of the OECD Test Guideline 201 (Micro-Algae, Growth Inhibition Test) has been underway. Since algal tests represent a special testing field and are different in many ways from the traditional ecotoxicological test methods for aquatic organisms, the updated OECD Test Guideline 201 should be consulted, once available, when specific guidance is needed for handling of difficult substances in algal tests.

Table 1. Relevant OECD Ecotoxicity Test Guidelines

Guideline number	Description of test
201	Alga, growth inhibition test
202 (updated draft)	*Daphnia* sp. acute immobilisation test
203	Fish acute toxicity test
204	Fish prolonged toxicity test
210	Fish early life-stage toxicity test
211	*Daphnia magna* reproduction test
212	Fish, short-term toxicity test on embryo and sac-fry stages
215	Fish juvenile growth test
219 (draft)	Sediment-Water Chironomid Toxicity Test Using Spiked Water
Draft	*Lemna* sp. growth inhibition test

Table 2. Properties of "difficult" substances and mixtures

Property	Nature of difficulty
Poorly water-soluble	• Achieving/maintaining required exposure concentrations • Analysing exposure
Toxic at low concentrations	• Achieving/maintaining required exposure concentrations • Analysing exposure
Volatile	• Maintaining exposure concentrations
Photo-degradable	• Maintaining exposure concentrations • Toxicity of breakdown products
Hydrolytically unstable	• Maintaining exposure concentrations • Toxicity of breakdown products
Oxidizable	• Achieving, maintaining and measuring exposure concentrations • Toxicity of modified chemical structures or breakdown products
Subject to corrosion/ transformation	• Achieving, maintaining and measuring exposure concentrations • Toxicity of breakdown products
Colloids	• Achieving, maintaining and measuring exposure concentrations • Light attenuation/scatter
Biodegradable	• Maintaining exposure concentrations • Toxicity of breakdown products
Adsorbing	• Maintaining exposure concentrations • Analysing exposure
Complexing	• Distinguishing complexed and non-complexed fractions in media • Depletion of nutrients in media
Coloured	• Light attenuation
Hydrophobic	• Maintaining exposure concentrations
Ionised	• Distinguishing ionised and non-ionised fractions in media • Defining exact exposure concentrations
Multi-component substances and preparations	• Preparing representative test media • Defining exact exposure concentrations

1. Preliminary assessment of stability of test substance

Modifications to media preparation and exposure systems may be required where exposure concentrations of a test substance are likely to decline significantly over the test period. In OECD guidelines for aquatic toxicity testing a decline in concentration of 20% is considered sufficient to warrant consideration of measures to reduce the decline. Data providing an indication of the stability of the test substance under the test conditions should therefore be obtained before commencing testing. Two potential sources of these data are:

- review of existing data on the physical and chemical properties of the substance; and
- data determined from a preliminary stability study carried out under test conditions.

1.1 Review of existing data on the test substance

Data relating to the physical/chemical properties, fate, transport and environmental toxicity of a test substance will be of assistance in selecting a media preparation regime and exposure system which will control the decline in exposure concentration. An example of a data profile which might be useful to assess these parameters is given in Table 3. The table includes values for some of the properties which are likely to be indicative of presenting technical difficulties for testing.

Existing reference sources or predictive methods (see Annex 1 for examples of computer-based predictive methods) should be used to construct the profile. The profile can then be reviewed with respect to standard media preparation and testing procedures and modifications identified and implemented prior to commencement of testing.

Table 3. Data profile for review of test substance properties and indicator values of difficulties for media preparation and testing

Property	Indicator value [1]
Physical state: liquid, solid, gas	-
Compositional information and chemical structures	-
Molecular weight(s)	>300 g/mol [2]
Is the substance ionisable? If yes, dissociation constant (pKa)?	-
n-Octanol/water partition coefficient (log K_{ow})	>4
Water solubility in test media under test conditions	< 100 mg/l
Water solubility in pure water at 25°C	< 100 mg/l
Critical micelle concentration	-
Vapour pressure at 25°C	-
Henry's Law Constant (H)	>0.1 Pa.m^3/mol
Boiling point	-
Melting point	-
Solubility in organic solvents	-
Water dispersibility for surfactants/detergents	-
Complexation constants	-
Time for ultimate aerobic biodegradation	<10 days [3]
Time for primary aerobic biodegradation	<5 days [3]
Soil adsorption coefficient (log K_{oc})	-
Hydrolysis half-life at 25°C and pH=7	<24 hours [4]
Fish bioconcentration factor (BCF)	> 500
Volatilisation (half-lives) from water: model river and lake	-
Sewage (wastewater) treatment plant removal percentage	-
Photodegradability	Absorbs light at wavelengths 290 - 600 nm
Toxicity to aquatic species	<1 mg/l
Dissolution rate and extent for metal compounds	-

1. Indicator values are not criteria of difficult-to-test substances. They indicate whether these substances are likely to present technical difficulties for testing.
2. Experience indicates that many difficult-to-test substances appear to have molecular weights > 300 g/mol.
3. This test is not routinely performed for many chemicals. The value depends on test duration.
4. The value depends on test duration.

1.2 Preliminary stability study

A number of physical, chemical and biological processes can result in significant declines in actual exposure concentrations of a test substance in aqueous media over time (see Table 2 and later sections). Where data are absent or insufficient to identify the process responsible for the decline it may be appropriate to carry out a preliminary study for assessing the stability of the test substance.

An example of a design for such an experiment is given in Figure 1. Solutions of the test substance are prepared under conditions equivalent to those to be used in the toxicity test (including aeration if that is envisaged), but excluding test organisms. Stable dispersion and emulsions of the test substance can only be used in exceptional cases (see Section 3.1.2 on "Dispersions and emulsions"). Samples of the test media are analysed at 0, 24 hours and at the end of the test or at a shorter period for unstable and adsorbing substances. A saturated solution need not be used for this test but it should contain a detectable concentration of the test substance. A sufficiently sensitive analytical method is necessary for the analysis of the test substance in the test medium. The possibility of losses during sampling, sample

treatment and analysis must be considered. Where samples are stored before analysis, losses during sample storage should also be assessed. If the test substance is so sparingly soluble that it cannot be detected using reasonable analytical techniques it will normally be necessary for the experimenter to use whatever physical-chemical data are available in conjunction with expert judgement in order to assess stability. Moreover, if a substance is so sparingly soluble that it cannot be detected, it is unlikely to be used in an aquatic toxicity test without a dispersant/solvent, and there would be no need to establish its stability without these. In exceptional circumstance it may be justified to use specialised techniques (e.g. radiolabelled test substance) in order to achieve quantitative assessments. The ^{14}C identity of the radiolabelled substance should be verified at the beginning and during the study. It may be noteworthy to mention that even if the radioactivity level is maintained, test substance can still be lost from solution.

The significance of volatility can be assessed by comparing concentrations in open and closed vessels. Similarly, the significance of adsorption onto test vessel surfaces can be assessed by a comparative study of concentrations determined in vessels conditioned to reduce adsorption and in unconditioned vessels. Methods to reduce volatile losses and partitioning onto test vessels are described in Sections 3.4 and 3.6.

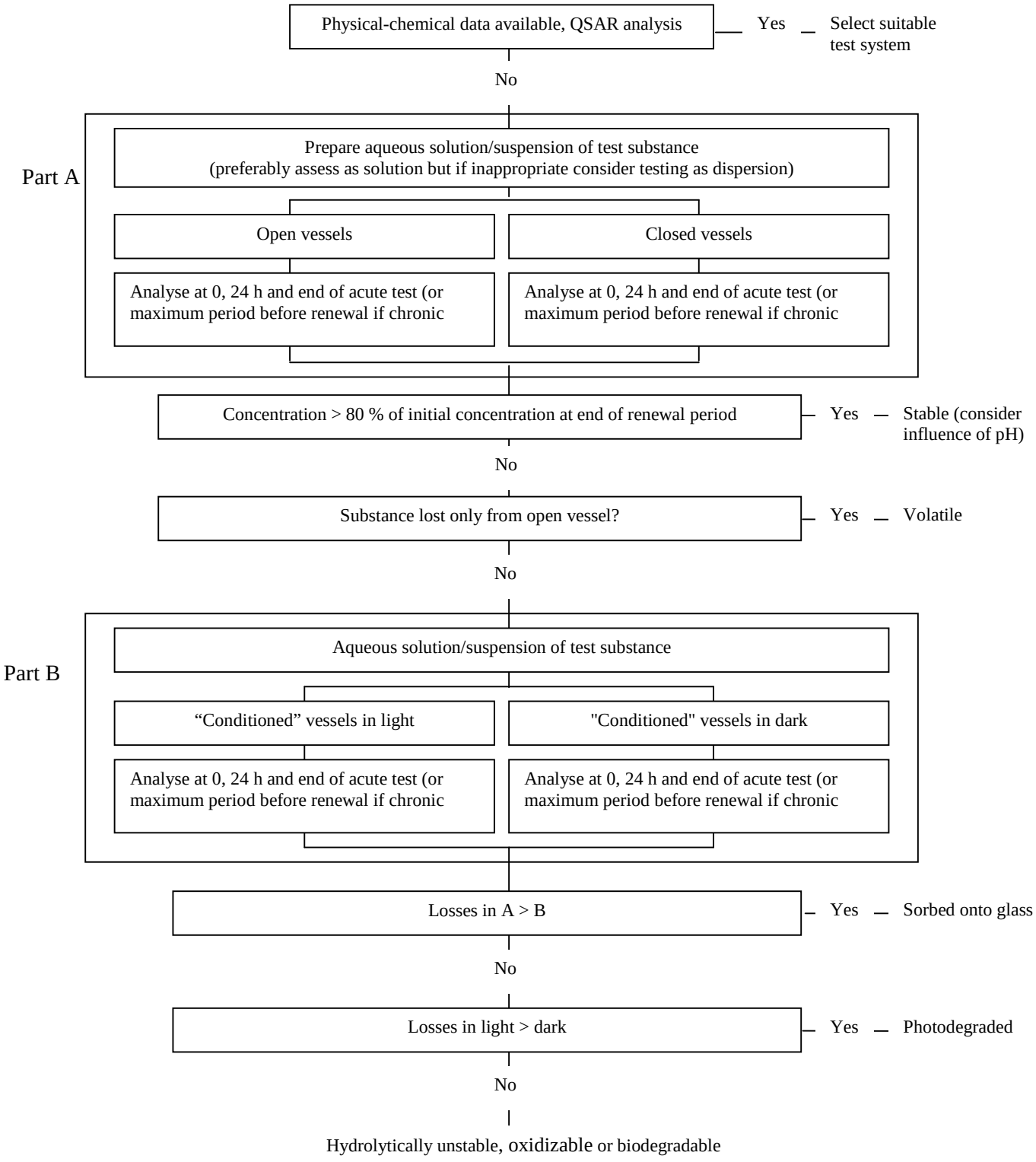
The significance of adsorption onto test vessel surfaces can be assessed by a comparative study of concentrations determined in closed vessels conditioned to reduce adsorption (see Part B of Figure 1) and in unconditioned closed vessels (see Part A of Figure 1). Methods to reduce partitioning onto test vessels are described in Sections 3.6.

If conditioning of test vessels has no appreciable impact on maintaining measured chemical concentrations, then it is most likely that the loss process is either photolysis, biodegradation or hydrolysis. However, it may also be due to oxidation. Photodegradation can be distinguished from a comparison of analysis of media held under conditions of light and darkness. Greater losses in the light point to photolysis whilst similar losses in both vessels suggest that hydrolysis or biodegradation are the more important loss processes. Methods to reduce losses by these routes are outlined in Section 3.5.

The design of the preliminary stability study may need to be tailored on a case-by-case basis to address other mechanisms responsible for reductions in exposure concentration, e.g. sorption onto organic food substances. Further guidance on the design and conduct of preliminary assessment studies can be found in documents published by the EC (EC, 1996b) and the US EPA (US EPA, 1996a); Nabholz (1991); Nabholz *et al* (1993); Auer *et al.* (1990); Lynch *et al.* (1994); Boethling and Nabholz (1997); and Newsome *et al.* (1996). The preliminary stability studies were not intended to be limited to laboratory studies.

It may be necessary to run the preliminary stability study alongside a preliminary assessment of toxicity, if stability is only of concern at concentrations that will be used in the toxicity test. Those concentrations may not be known beforehand and they may affect the stability of test substances in the test media. For example, complexation may occur at higher concentrations and losses may be greater (proportionally) at low concentrations than at high concentrations. It may be necessary to carry out the preliminary study at least with two concentrations to identify these problems.

Figure 1. Design of a preliminary experiment for identifying process(es) responsible for loss of test substance from aquatic toxicity test media



2. General considerations on selection of exposure regimes

The selection of the exposure regime to be used in aquatic toxicity tests should be guided by the time course of the experiment, the test species used, and the characteristics (e.g. physico-chemical properties) of the test substance and/or the results of a preliminary stability study. The latter is conducted to determine the likely fate and behaviour of the substance under very similar test conditions as those prevalent in the actual aquatic toxicity test. The exposure regime could be:

- Static, no replacement - the test medium is not replaced for the duration of the study;
- Semi-static replacement - the test medium is periodically replaced on a batch basis;
- Intermittent flow-through replacement - the test medium is replaced over set periods during the exposure; and
- Continuous flow-through replacement - the test medium is continually replaced.

Note: For some chemicals, especially crop protection products, pulsed or time-varying exposure designs have been developed.

It is not possible to give definitive guidance on criteria for deciding on exposure regime since requirements may vary between different regulatory authorities. However, a static exposure regime is likely to be appropriate if exposure concentrations can be expected to remain within 80-120% of nominal over the whole test period without renewal of the test media. Likewise, a semi-static or intermittent or continuous flow-through regime will probably be required where concentrations are unlikely to remain within 80-120% of nominal. Where uncertainty exists over the suitability of an exposure regime it is good practice to consult with the appropriate regulatory authority prior to commencement of the test.

Renewal of test solutions after 24 hours exposure will frequently allow exposure concentrations to be maintained and is a relatively simple modification to the test procedure. More frequent semi-static renewal is possible but care must be exercised not to excessively stress fragile test organisms, e.g. daphnids, by excessive handling. An intermittent flow-through regime may reduce the stress imposed on test organisms compared with a semi-static regime and systems for use in acute and chronic tests with daphnids and early life-stage tests with fish have been referred to by Van Leeuwen *et al.* (1986). A continuous flow-through regime should generally be considered when a semi-static regime with renewal following each successive 24 hours exposure period is incapable of maintaining exposure concentrations. Flow-through test systems are discussed further in Section 3.1.3 and 3.4 in particular.

Semi-static renewal and flow-through regimes are likely to require frequent cleaning of the exposure systems to prevent the accumulation of organic debris and the development of excessive microbial populations. Care should be taken to minimize stress on test organisms caused by cleaning.

It should be stressed that flow-through systems cannot be used for algal testing.

3. Media preparation and exposure systems for difficult test substances

The objective in preparing test media should be to achieve the required exposure concentrations of dissolved test substance at the start of the test. These concentrations should then be maintained, where technically possible, throughout the test using either static, semi-static renewal or flow-through exposure regimes.

The method used for preparing aqueous stock or test solutions is dictated by factors such as the physical state of a test substance, its solubility in the test dilution water and the desired range of test concentrations relative to the solubility of the substance. All reasonable efforts should be made to achieve true test solutions containing dissolved concentrations of the test substance that are stable and close to target. However, it has to be recognised that actual exposure concentrations can be substantially lower than nominal concentrations as a consequence of a number of loss processes. Hence, while the target should be to achieve exposure concentrations up to the aqueous solubility of the test substance, it may be necessary to utilise higher nominal concentrations to achieve it. Visible non-solubilized test substance in the exposure vessels is generally to be avoided or minimized. Exceptions are possible when suitably justified (e.g. see two exceptions in Section 3.1.2 on “Dispersions and emulsions”).

The media preparation and exposure methods should be evaluated prior to commencing testing to ensure that the required quantities of test medium can be produced and the required concentrations can be achieved and maintained. Consideration should also be given to testing a reference substance in order to establish that the methods achieve results comparable to those obtained using standard methods. Approval for non-standard methods should be sought from the appropriate regulatory authority and the methods should be fully described in the test report.

It may be noteworthy that for assisting classification of metals and metal compounds, an OECD protocol on transformation/dissolution of metals and metal compounds is currently being developed.

3.1 Poorly water-soluble substances

3.1.1 Preliminary experiment

A preliminary experiment should be considered for all studies with poorly water-soluble substances to determine the maximum dissolved concentration that can be achieved under test conditions, which is defined as the saturation concentration, and the preparation conditions that are required to achieve it. The results of the preliminary experiment will form a basis of and justification for the media preparation procedures adopted for the toxicity tests and a reference point (i.e. water solubility under test conditions) against which the test results can be evaluated. In view of its importance the preliminary experiment should be fully reported.

Solutions of test substance could be prepared at higher nominal concentrations than the theoretical water solubility limit to achieve the maximum dissolved concentration provided any non-dissolved substance is separated before testing. This method is also useful to take into account soluble impurities.

The design of the preliminary experiment should take into account the possible need to remove non-dissolved test substance from the test medium. It should not be assumed that a clear test medium

indicates a true solution since crystals, aggregates, micelles etc. cannot easily be detected by visual observation. Possible separation techniques include:

- Centrifugation - the preferred separation method but there may be practical difficulties in applying the technique to large volumes of test medium. As a guide, centrifugation at 100,000 to 400,000 $m.s^{-2}$ for 30 minutes may achieve adequate separation. It should be noted that most centrifuge containers are made of various sorts of plastics which may adsorb the test substance, and that glass containers are more likely to break.
- Filtration through a membrane filter - less widely advocated because of the potential for losses due to adsorption onto the filter matrix. Filtration may represent the only practical option where large volumes of test media are required. Filter sizes of 0.22 to 0.45 μm may be suitable for achieving adequate separation. Filter matrix should be made of inert materials (i.e. chemically and physically non-reactive with testing compounds). Filters should be rinsed with high-purity water prior to use to reduce the risk of contamination of test solutions with toxic residues. Adsorption of the test substance may be reduced to insignificant levels by preconditioning filters with solutions of the test substance prepared at the appropriate test concentrations. Filtration under pressure is preferable to vacuum filtration due to potential losses by evaporation.

A justification for, or validation of, the separation technique should always be provided in the test report. Separation techniques should not be applied to surfactant solutions.

It should be noted that a high level of uncertainty is often associated with solubility estimates for poorly water-soluble substances. It is therefore often not possible to specify an upper exposure limit with a high degree of confidence. Exposure concentrations which exceed a "reported" water solubility should therefore not necessarily be considered invalid but reviewed on a case-by-case basis.

3.1.2 Media preparation methods

Four methods of test media preparation have been applied to poorly water-soluble substances. These methods are referred to here by the generic terms: direct addition, water-miscible solvents, generator systems and dispersions and emulsions. Care should be taken due to the fact that stability of a substance measured in the laboratory can differ greatly from that observed in the test system.

Direct addition

Direct addition, as the term implies, involves the bulk addition of a test substance to the water. It will usually be necessary to mix the substance with the water to ensure its dispersion through and solution in the water column. The effectiveness of direct addition is therefore very dependent upon using a mixing regime appropriate to properties of the substance such as viscosity and density. The following mixing techniques are identified below in increasing order of severity:

- low energy stirring;
- vigorous shaking;
- blending;
- homogenisation/high-shear mixing;
- ultrasonication.

For stirring, a 48-hour period might be considered reasonable to achieve maximum solubility, although a slightly higher concentration might be achieved by stirring for 6 weeks. A period of 30 minutes has been suggested for ultrasonication, but this could lead to considerable heating, particularly of small volumes, and potential consequences for stability.

Mild heating of the substance and water may also help to achieve more rapid solution of a test substance but care must be taken to ensure that this does not result in changes to either the test substance or the water which could compromise the outcome of the test. Attention should also be given to potential decrease of dissolved oxygen concentration.

It is recommended that use of direct addition should be limited to the cases when the concentrations to be reached are lower than 50% of the water solubility. However, direct addition by weighting may be difficult when testing a substance with very low solubility at low concentrations, because making a large volume of a very diluted solution may inherently be uncertain as regards whether or not microparticles may be present and thus included or not when using a small fraction of the solution. Although direct addition of a suitable volume of a poorly soluble test chemical is the preferred method, in practice it may be impossible for very toxic chemicals. In general, it is not possible to weigh out an amount less than 5-10 mg with a sufficient precision. The preparation of a test solution with a nominal concentration of less than 1 mg/litre will thus require more than 10 litres of the test medium for dispersion, and a test concentration of 0.1 mg/litre will similarly require more than 100 litres. It is not possible to handle such large volumes in practice. Therefore, serial dilution of the Water Accommodated Fraction (WAF, see Section 3.11) of a properly prepared stock solution may be the only applicable method for such very toxic chemicals.

Solvents

Water-miscible solvents provide a vehicle in which some poorly soluble substances can be dissolved to produce a stock solution which is more amenable to adding to, and mixing with, the test media. In particular, solvents could be helpful for hydrolytically unstable and highly viscous substances. However, because of the potential for interaction with the test substance resulting in an altered response in the test, their use should be restricted to situations where no other acceptable method of media preparation is available. If solvents are used, their effects on the test results, if any, need to be determined. It should be emphasised that solvents are not appropriate for mixtures where the use of the solvent can give preferential dissolution of one or more components and thereby affect the toxicity. This could also be true where the technical grade of a substance is tested and a toxic impurity dissolves preferentially.

The choice of solvent will be determined by the chemical properties of the substance. Solvents which have been found to be effective for aquatic toxicity testing include acetone, ethanol, methanol, tertiary-butyl alcohol, acetonitrile, dimethyl formamide and triethylene glycol. The physico-chemical properties and aquatic toxicity of a range of solvents have been reviewed by Tarr and Hutchinson (1992) and ECETOC (1996). It should be noted that the use of solvents such as acetone, ethanol and methanol can be problematic for static and semi-static systems due to substantial growth of bacteria in aquatic test systems and resulting depletion of oxygen. Caution should be exercised to avoid critical oxygen depletion in long term tests with intermittent or continuous flow-through regimes.

The recommended approach to media preparation is to prepare a concentrated stock solution of the test substance in the solvent. Measured quantities of the stock solution are then added gradually to the test dilution water whilst continuously mixing using an appropriate technique. The concentration of the solvent in the final test medium should not exceed the corresponding toxicity thresholds determined for the solvent under the test conditions. The suggested level is at least one order of magnitude below the appropriate no-observed effect concentration (NOEC) depending on the test species and the length/type of toxicity test or in any case below 100 mg/l (or 0.1ml/l). In practice, a concentration of 100 mg/l (or 0.1 ml/l) is a reasonable working maximum concentration for most of the commonly used solvents. It is unlikely that a solvent concentration of 100 mg/l will significantly alter the maximum dissolved concentration of the test substance which can be achieved in the medium.

The use of volatile solvents (n-hexane and petroleum ether) which can be stripped from the medium after mixing has also been referred to in ISO (1997). The potential for co-stripping of volatile test substance from, and the retention of toxic solvent residues in, the test medium are likely to restrict the applicability of this approach.

OECD guidelines for aquatic toxicity tests require that the concentration of the solvent must be the same in all treatments, where technically feasible in flow-through systems, and that the test design includes a control group of organisms which are exposed to this concentration.

Generator systems

Aqueous solutions of a poorly soluble pure substance can be produced by bringing water into contact with a chemically inert matrix which has been coated with the substance. A matrix with a high surface area to volume ratio (such as pumice or glass beads) allows a relatively large amount of test substance to be coated on the surface and provides a large area over which dissolving of the test substance can take place. Systems utilising columns packed with a suitable matrix have been described by Veith and Comstock (1975), Gingerich *et al*. (1979) and Phipps *et al.* (1982). The aqueous solution drawn off from the column can be used either directly for testing or diluted to produce a concentration series.

Generator systems based on semi-permeable membrane disks (e.g. Empore™) offer an alternative to column-based systems and have the advantage of ensuring that only dissolved material is present in the resulting test medium. A system which utilises a disk placed in the headspace has been described by Urrestarazu Ramos *et al*. (1997).

It may be necessary to re-circulate a test medium repeatedly through a column or disk in order to achieve a saturated solution. Where the test medium is recycled it may also be necessary to re-circulate in order to replenish losses due to sorption or bioaccumulation. The latter practice may result in complexation of the test substance with dissolved organic matter and measured total concentrations of the test substance exceeding the saturation concentration (Billington, 1988).

Generator systems are not considered appropriate for substances containing components or impurities which differ in their water solubility. Differences in water solubility will result in selective depletion of the more water soluble components from the column or disk matrix and their relative concentration in the water phase. Generator systems are also not suited to substances which are hydrolytically unstable since the composition of the derived test medium is likely to change over time. Another disadvantage of the generator column method is the difficulty in obtaining large volumes of solution which makes their use in flow-through systems problematic.

Dispersions and emulsions

The testing of aqueous dispersions and emulsions is not generally advocated for the following reasons:

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

There are two exceptions to this rule:

- Where there is a regulatory requirement, such as assessing oil dispersing agents, pesticides which are formulated for use as a dispersion or emulsion, and industrial chemicals which are emulsified for use and released as an emulsion, such as some polycationic polymers with a silicone polymer backbone. Guidance on the testing of substances in the presence of chemical dispersing or emulsifying agents is best sought from the appropriate regulatory authority;
- Where the test substance has an inherent tendency to form an aqueous dispersion or emulsion such as surfactants and detergents. The highest test concentration should either be 1000 mg active ingredients/litre or the dispersibility limit (i.e., the limit at which phase separation takes place), whichever is lower.

Stable dispersions or emulsions can sometimes be produced by the simple expedient of physically mixing the test substance with the aqueous phase. Appropriate solvents used in conjunction with physical mixing may also be effective, particularly where the test substance is a solid. 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. The potential for any method of dispersion or emulsification causing breakdown of the test substance should be checked before using it in the toxicity test.

A dispersion or emulsion should be stable before the test organisms are introduced. Any excess of test substance not uniformly distributed throughout the test medium should be removed. No attempt should be made to maintain test material in suspension, by for example stirring, except where this is required by specific test protocols. A semi-static replacement regime should be considered if a stable dispersion or emulsion cannot be maintained for the duration of the test.

The potential for non-dissolved test material to cause physical effects on the test organisms should be estimated during the test. Physical effects, such as the blocking of fish gill membranes, the encapsulation/entrapment of daphnids or the reduction of light intensity in the algal test, can lead to an overestimation of toxicity. Techniques for physically separating the test organisms from non-dissolved material, whilst maintaining contact with the water column, should be considered where physical effects are likely to be significant (see Section 3.9 on “Hydrophobic substances”).

In tests with dispersions or emulsions of pure single substances, exposure concentrations should be expressed in terms of the concentration of the test substance in solution, otherwise the toxicity may be underestimated. However, there are some exceptional cases. For example, where the pure substances are self-dispersing in water forming micelles, micro-dispersions and macro-dispersions such as certain surfactants, some charged polymers, many aliphatic amines, exposure concentrations should be expressed in terms of the whole test substance dispersed in dilution water. It is therefore recommended to consult the regulatory authority to decide how exposure concentrations are to be expressed. Dissolved concentrations can be approximated by measurement following treatment to separate non-dissolved material from the aqueous phase (see Section 3.1.1).

Toxic effect concentrations for dispersions and emulsions should be compared with the dispersibility limit or the critical micelle concentration for a substance in water rather than with its water solubility limit.

3.1.3 Flow-through exposure systems

Substances which are solids at room temperature may take a significant amount of time to reach their solubility limit, even when added as a solution in a water-miscible solvent and subject to vigorous

mixing. Consequently a flow-through system, using a solvent stock solution of the substance for dosing the medium, may not give sufficient time for the substance concentration to equilibrate. Under such circumstances it may be appropriate to prepare, in advance, sequential batches of test solution sufficient for a period of e.g. 24 hours or longer as necessary, which can be pumped directly into the test vessels. It should be noted that such solvent systems are not appropriate for metals.

3.1.4 Culture and test media - considerations when testing metals and metal compounds

Media specified for culturing and testing with invertebrates, plants and algae may contain concentrations of some essential elements, such as copper, zinc and nickel, that are only just sufficient to meet nutritional requirements. As nutritional requirements depend on the acclimatisation history of the organisms, ecologically relevant culture conditions need to be used. The composition of culture and test media may therefore require special consideration to ensure that test results correctly reflect the toxicity of metals and metal compounds.

3.2 Substances that are not acutely toxic at the limit of their water solubility

It is important to recognise that the maximum achievable dissolved concentration of a substance in the test medium, i.e. saturation concentration, may not be the same as the water solubility of the substance as determined by, for example, OECD Guideline 105. Typically, the concentration will be less. It is also important to note that water solubility measurements made for regulatory purposes are usually made in distilled water (pH=6-9) and not test media (pH=7-8) and that differences in pH of the test media and distilled water may significantly affect the solubility, especially of ionised substances with a pKa between 5 and 9.

If it is necessary to assess the toxicity of a substance at the saturation concentration, it is important to provide evidence that all reasonable efforts have been taken to achieve this. The evidence might include:

- the report of a preliminary experiment demonstrating that the media preparation regime is sufficient to maximise the concentration of the test substance in solution (see Section 3.1 “Poorly water-soluble substances”);
- a description of the method of media preparation regime used for the test;
- a statement of the water solubility; and
- an assessment of saturation concentration.

An effect concentration can be measured only if it is equal to, or less than, the saturation concentration. If an effect cannot be detected in a test with a saturated solution the result should be reported as no toxic effects at saturation. It is important to note that an absence of acute toxic effects at the saturation concentration cannot be used as the basis for predicting no chronic toxicity at saturation or at lower concentrations. Where chemicals are predicted to have no acute toxic effects at saturation, it is recommended to consult the regulatory agency. Some regulatory authorities may prefer to omit acute toxicity tests and proceed straight to chronic toxicity testing.

3.3 Substances toxic at low concentrations

Serial dilution of stock solutions provides a mechanism for adding small quantities of test substance. Stock solutions may be prepared in water or a water-miscible solvent as appropriate (see

Section 3.1). Effective mixing is essential to ensure that the test substance is uniformly dissolved throughout the test medium.

Exposure concentrations should ideally be confirmed and their stability demonstrated by analysis. The use of a radio-labelled test substance may enable very low concentrations to be analysed but this may lead to significant additional costs associated with its synthesis, handling and analysis. In the absence of a suitable analytical method for quantifying exposure, a semi-static renewal or flow-through regime may be necessary to ensure that exposure concentrations are in line with target values.

Where measured concentrations decline significantly over the exposure period, the geometric mean concentration should be used as the basis for assessing effects. However, it should be noted that several types of loss processes (e.g. precipitation) can occur very fast and new equilibrium conditions can be attained relatively quickly during the exposure. If this was the case, the median of the concentrations which are measured after the decline would be more appropriate as a surrogate for the mean exposure concentration. Where a measured concentration at the end of the exposure period is absent or where it indicates that the substance is not detected, the validity of the test should be reconfirmed. In order to calculate a mean exposure concentration, the final concentration may be taken as the limit of detection for the method if the substance is not detected. When the substance is detected but not quantified, it may be a good practice to use half of the limit of quantification. Since there may be various methods for determining that, the method selected to determine mean measured concentrations should be made explicit in the reporting of test results.

3.4 Volatile substances

The vapour pressure (*vp*) and Henry's law constant (*H*) of a substance are important parameters determining the potential for a substance to be lost to the atmosphere by evaporation.

Vapour pressure is a measure of the equilibrium between the condensed and vapour phases of a substance. Vapour pressure is relevant to aquatic toxicity testing where non-dissolved test substance on the surface of the medium is in contact with the atmosphere. Under this condition transfer to the vapour phase alters the equilibrium between the dissolved and non-dissolved phases and changes the composition of the medium. The significance of losses from the medium via this route can be calculated assuming ideal gas behaviour and the volume of a mole of substance at the temperature and pressure of the system.

The Henry's law constant for a substance is a measure of its equilibrium between an ideal solution phase and the vapour phase. As such it is a measure of the potential for a substance to be lost from solution by evaporation. Molecular weight (*MWt*, g/mol), water solubility (*S*, mg/l) and vapour pressure (*vp*, Pa) can be used to estimate Henry's law constant (*H*, $Pa.m^3/mol$) from the relationship:

$$H = \frac{vp \times MWt}{S}$$

As an approximation, if *H* is greater than 100 $Pa.m^3/mol$, more than 50% of the substance could be lost from the water phase in 3-4 hours (Mackay, 1992). However, other factors in the test system may affect the rate of loss, principally test vessel size and shape, depth and temperature of the medium and rate of aeration. The losses due to volatilisation may become significant for substances with Henry's law constants of 1-10 $Pa.m^3/mol$ under vigorous mixing conditions where the opportunity for water/air exchange is high.

Losses of volatile substances from test media during preparation and exposure can be minimised using relatively straightforward modifications to procedures. As a general rule vessels should be sealed during preparation and exposure and the headspace kept to a minimum. Likewise, test concentrations should, where possible, be prepared individually by addition of test substance directly to the test vessels rather than by dilution of a stock solution. Systems with zero headspace should be used where it is not possible to analyse exposure concentrations. Syringe pumps can be used to dispense concentrated solutions of volatile substances (in water miscible carrier solvents, if required) into sealed vessels. Samples collected for analysis should be placed in zero headspace vials.

A tiered approach, along the lines of the following, is suggested for selecting an appropriate exposure system:

1. Open system, no test medium renewal, analytically determined exposure concentrations;
2. Open system, static renewal of test media, analytically determined exposure concentrations;
3. Open system, flow-through medium replacement, analytically determined exposure concentrations;
4. Closed semi-static renewal or continuous flow-through system, with or without headspace, analytically determined exposure concentrations; and
5. Closed semi-static renewal or continuous flow-through system, no headspace, nominal exposure concentrations.

Selection of the appropriate system should be dictated, where possible, by the goal of maintaining analytically quantifiable test substance concentrations throughout the test.

Ideally, the maximum exposure concentration should not exceed the water solubility of the substance. If it is not the case, the approaches adopted should be consistent with those described in Sections 3.1 and 3.11 dealing with “Dispersions and emulsions” and “Multi-component substances”, respectively.

The following comments relate to specific tests:

- Fish tests: Depletion of dissolved oxygen from media in sealed vessels may be significant in fish tests. Consideration should therefore be given to using fish at the smaller end of the preferred size range, larger test volumes, aeration with pure oxygen or more frequent medium renewal (semi-static or flow-through) in order to maintain oxygen concentrations within guideline values. When using pure oxygen, the pH of the test solutions should be monitored. Dosing systems for flow-through tests have been described by Mount and Brungs (1967) and Benville and Korn (1974).

- *Daphnia* reproduction test: Removal of progeny and renewal of the test media should be undertaken at the same time in the 21-day *Daphnia* reproduction test to minimise the number of occasions when the test vessels are unsealed. Depletion of dissolved oxygen is not generally a factor in *Daphnia* tests providing test volumes are not too small and the test media are changed relatively frequently e.g. daily. Flow-through exposure systems for *Daphnia* chronic tests have been described in ASTM (1997), Diamantino *et al*. (1997), Sousa *et al*. (1995) and US EPA (1996b).

- Algal test: Algal tests with very volatile substances are technically very difficult to perform satisfactorily and may as a consequence yield results that are difficult to interpret and of limited relevance to real-world conditions. Guidance for algal growth inhibition tests has been given in ISO (1998). The use of a sealed exposure system in the algal growth inhibition test will result in culture growth being limited by CO_2 depletion and increasing pH. Consideration should therefore be given to reducing the inoculum cell density and adding additional sodium bicarbonate to the medium. Alternatively, a flow-through system capable of maintaining the supply of CO_2 whilst avoiding a

headspace could be considered. Such a system has been developed by Tjeerdema and Singer (1991) but has yet to be described in an internationally or nationally agreed test guideline. Halling-Soerensen *et al.* (1996) have described a system for testing volatile substances which utilises a CO_2 enriched headspace and Mayer *et al*. (2000) described a system using completely closed flasks with no gas phase in which gaseous CO_2 is simply replaced with $NaHCO_3$ and pH is adjusted with HCL. Whatever system is adopted it is advisable to establish that acceptable control culture growth can be achieved before starting the test. A test with a reference substance should also be performed to confirm that the result is consistent with that obtained in a conventional test system. The test result should be reported with the reference substance.

3.5 Substances that degrade in the test system

The preliminary stability assessment study outlined in Section 1.2 should consider the stability of the test substance in water. If the substance is likely to be unstable, a decision to test the parent substance and/or its degradation products, if identified, should be based on a consideration of its half-life under test and real-world conditions. The following decision criteria are suggested only as a guide for static and semi-static tests with medium renewal times of 24 hours:

- Half-life >3 days: test parent substance;
- Half-life <3 days and >1 hour: consider on a case-by-case basis, and include possible testing of degradation products;
- Half life <1 hour: test degradation products.

These criteria are based on the assumption that problems associated with maintaining exposure concentrations are often more noticeable in static and semi-static renewal tests compared with flow-through tests. Loss of 20% of the initial concentration of a substance over a 24-hour period corresponds with a half-life of approximately 3 days. A half-life of <3 days is therefore likely to result in exposure concentrations decreasing to below target values and a possible build-up of degradation products over 24 hours.

Testing of both degradation products and parent compound depends on the objectives and regulatory requirements.

The results of a preliminary toxicity test in which test organisms are initially affected and then recover may indicate that the parent substance is more toxic than its degradation products. In this case the toxicity of the parent substance should be determined, even if exposure levels cannot be maintained to the extent necessary to comply absolutely with test guidelines. It should be noted that some regulations also require the toxicity of the parent substance to be determined.

Testing of degradation products will normally be required where the results of a preliminary range-finding experiment or a (Q)SAR analysis indicates that they have significant toxicities or other relevant properties (e.g. low or no degradability). The aquatic toxicity of degradation products may be determined by allowing the parent compound to degrade and then exposing the test organisms to the resulting media. Leaving a stock or test solution of the parent substance for a period equal to 6 half-lives of the substance will generally be sufficient to ensure that the medium contains only degradation products. The pH of the degraded solution should be neutralised to that of the control medium prior to testing.

Test results obtained for non-degraded and degraded solutions should initially be compared on the basis of the concentrations of the parent substance used in preparing the solutions. In this way the

relative toxicity of the parent substance and degradation products can be assessed. Identification and quantification of the degradation products may subsequently be necessary to aid in interpreting test results.

The choice of medium replacement regime in fish tests with degradation products needs to take into account water quality considerations as well as chemical and concentration stability. Where the degradation products have been clearly identified it may be preferable to determine their toxicity separately. The testing of degradation products imposes a requirement for an analytical method to determine their concentration. This will be additional to the method required to determine the parent substance. Samples taken for analysis should be treated and/or stored in an appropriate manner to prevent further degradation prior to and during analysis.

A useful bench-mark when designing flow-through exposure systems is that, for a substance with a half-life of 4 hours, approximately 50% of the nominal concentration should be able to be maintained using a system with 6 volume renewals in 24 hours. A flow-through system which delivers more than 6 volume replacements in 24 hours will therefore be required when it is necessary to achieve concentrations which are closer to nominal.

It should be noted that when selecting exposure regime and test vessels for unstable substances, the tiered approach suggested for volatile substances (see Section 3.4) is applicable. Selection of the appropriate systems should be dictated by the goal of maintaining analytically quantifiable test substance concentrations throughout the test.

3.5.1 Photolysis

In short-term acute fish and *Daphnia* tests breakdown of chemical structures by photolysis may be reduced or prevented by working in a darkened environment and using red light where necessary (ISO, 1997). It may also be possible to identify and selectively eliminate the light wavelength(s) responsible for photolysis. For longer-term chronic tests it is not advisable to carry out tests in complete darkness because of the risk of imposing additional stress by disrupting normal behaviour.

In algal tests it may be possible to determine the toxicity of the parent substance using one of two approaches:

- The first approach is based on selective removal from the illumination source of light wavelengths responsible for photolysis whilst retaining those wavelengths necessary for photosynthesis. However, this is not often workable since in most cases information on the wavelengths that are responsible for photolysis is not available. Although the UV spectrum may be available, the particular wavelengths causing degradation are unlikely to have been identified. It is also worth considering that, in the majority of cases, it is high energy wavelengths that give rise to photodegradation and these would not pass through borosilicate glass flasks. When considering such an approach it is important to recognise that algal tests should be performed at light saturation (about 100 $\mu E/m^2/s$ for species of green algae). Increased illumination may be required to compensate for the reduction in photosynthetic light when filters are used to remove light wavelengths responsible for photolysis. Appropriate controls should be included in the experiment design to demonstrate that illumination conditions are capable of sustaining acceptable algal culture growth.
- The second approach involves carrying out the test using a dark exposure phase followed by an illuminated phase. In this case interpretation of the test result will only be straightforward if the parent substance is algicidal, since once illuminated the potential exists for both the parent substance and the degradation intermediates to exert their effect(s).

However, it should also be mentioned that these approaches have been questioned. Instead, performing an abiotic experiment and subsequently doing an algal test on the control, simply inoculating the abiotic control with algae as appropriate (or as in the test) has been proposed.

The use of flow-through exposure systems should also be considered for photo-degradable substances. Key considerations in their design include eliminating the potential for photo-degradation of stock solutions and selection of appropriate flow-through rates to limit the extent of degradation in the exposure vessels.

3.5.2 Hydrolysis

Exposure concentrations of the parent substance should be maximised by keeping duration of the media preparation stage to a minimum. Direct addition of the substance to the test medium combined with methods to achieve rapid dissolution are preferred. Where stock solutions are required, consideration should be given to preparing these using non-reactive water-miscible solvents to minimise hydrolysis prior to dosing test vessels (see Section 3.1.2).

Flow-through exposure systems are not considered appropriate for the small number of substances which hydrolyse at high concentrations to form polymers (e.g. alkyloxysiloxanes and isocyanates) because of the potential for fouling of the test organisms and apparatus. These substances should be added directly to the exposure vessels, vigorously mixed. All test organisms need to be added as quickly and safely as possible after test substance addition within the time-scale required to maintain the health of the control organisms, for example, 10 minutes. If the substance is too insoluble to achieve a homogeneous dispersion after vigorous mixing, it should first be diluted in a non-reactive solvent and then added directly to the exposure vessels and mixed. Once again the organisms should be added as quickly and safely as possible.

Solutions of hydrolysis products of substances which polymerise should be prepared by adding the substance very slowly to a vessel which is part-filled with water and being stirred rapidly. Once the substance has been added, the vessel should be topped up with water to the required volume and stirred continuously for a period sufficient to ensure complete hydrolysis. This procedure should enable a solution of the hydrolysis products to be produced without the formation of polymers.

Temperature and pH can influence the rate of hydrolysis of some substances. Adjustment of these parameters, within the range permitted for the test, may therefore be appropriate in order to optimise exposure concentrations of the parent substance. The significance of pH for the rate of hydrolysis should be determined and the result used (if required) to identify conditions for producing hydrolysis products for testing. Testing of hydrolysis products should be performed at the normal test medium pH.

3.5.3 Oxidation

Oxidation is an abiotic transformation process which can result in breakdown of chemical structure and consequent effects on the toxicity of a substance. In aquatic systems oxidation also reduces the amount of dissolved oxygen which is available for respiration by aquatic species. The term “reducing agents” is applicable to substances which are subject to oxidation.

Oxidation cannot be prevented in oxygenated aqueous media. Maintenance of dissolved oxygen concentration is therefore a key consideration for fish and invertebrate testing. Selection of a static, semi-static renewal or flow-through exposure regime should be guided by the need to maintain the dissolved oxygen concentration within the range permitted for the test. Maintenance of oxygen concentration may be facilitated by aeration of the medium, increasing the volume of test medium and/or reducing the test

organism loading or increasing the rate/frequency of test medium renewal. Flow-through test systems may often be appropriate. Stock solutions of the test substance should be kept under anoxic conditions (e.g. under nitrogen) until introduced to the test medium.

3.5.4 Biodegradation

Readily biodegradable test substances are likely to be degraded in aquatic test systems once competent bacterial populations become established. Maintenance of exposure concentrations is therefore dependent upon preventing the development of significant microbial populations.

Strict test vessel hygiene, at the start and during the course of the test, will delay and limit but not prevent the development of populations of bacteria capable of degrading the test substance. It is also important that carryover of old test medium is kept to a minimum and that test vessels are thoroughly cleaned and, where possible, sterilised at medium renewals. Antibiotic use is to be avoided. When they must be used, this should be defended by the sponsor and the testing laboratory. An antibiotic control will also need to be added to the experimental design.

A flow-through exposure regime with sufficient volume renewal and a high concentration stock solution maintained under nitrogen has been shown to: prevent aerobic biodegradation, minimise the concentration of breakdown products and maintain exposure concentration of the parent substance (Tolls *et al.*, 1997). Good hygiene procedures will also help to prevent the development of high bacterial populations on surfaces in the exposure system.

Care should also be taken to maintain dissolved oxygen concentrations, particularly in fish tests.

3.6 Adsorbing substances

In most cases adsorption onto surfaces of exposure systems and onto organic material will only be important where relatively low concentrations of a substance are being tested (e.g. <1 mg/l). The most common adsorption mechanisms responsible for losses are as follows:

- adsorption to test vessels (typically made of glass) via hydrogen or ionic bonding between the substance and hydroxyl groups on the vessel surfaces. Glass surfaces offer negatively charged hydroxyl groups which can bind with cationic substances such as surfactants. Salt bridges, formed by divalent cationic substances, such as Ca^{2+} and Mg^{2+}, may also be responsible for binding anionic substances;
- adsorption to negatively charged biological material, such as algal cells, by the same mechanisms described above.

The following approaches to reducing adsorption should be considered where losses of the substance are likely to compromise compliance with test guidelines:

- reducing the surface area to test medium volume ratio;
- increasing the frequency or rate of test medium renewal in semi-static and flow-through tests;
- construction of exposure systems using non-adsorptive materials such as polytetrafluoroethylene (PTFE);
- avoiding using highly adsorptive materials in the construction of exposure systems (in particular, rubber and polyethylene should never be used as part of exposure systems in any situation);
- pre-conditioning of test vessels using solutions of the test substance (N.B.: The concentration of the test substance used to condition a vessel should not exceed the test concentration appropriate to the

vessel otherwise the substance may desorb during the test and increase the exposure concentration. It should be noted that any flow through regime should always be equilibrated with the test substance, regardless of adsorptive substance testing or not, and confirmed by the pre-test samples on e.g. two days at least.);

- pre-conditioning of test vessels using silanising agents (e.g. a 5% solution of dichlorodimethylsilane in chloroform or heptane) [N.B.: many silanising agents are highly toxic and great care should be taken to ensure that residues are removed from the vessels before they are used in a test. A regime of repeated rinsing with water or baking at 180°C for 2 hours has been recommended (ISO, 1997). Organic solvents such as methanol could also be used in the rinsing steps];
- maintaining dissolved total organic carbon concentrations (other than that due to the test substance) in all tests at or below 2 mg/l, unless special studies are being conducted;
- removing excess food from test vessels after feeding has finished;
- feeding of fish a few hours before test medium renewal in semi-static tests; and
- using semi-static or flow-through exposure regimes instead of a static one.

Substances which sorb onto glass are usually very hydrophobic. Adsorption losses of test substance onto the surfaces of vessels used for analysis sampling can be prevented by priming the vessel with a small volume (usually 10-20 ml when a 50 ml sample is taken) of a water-non miscible solvent, such as hexane. Partitioning into the solvent phase is favoured at the expense of sorption onto the glass surfaces of the sampling vessel.

The influence of sorption on algal biomass in algal toxicity tests may be mitigated by reducing the inoculated algae concentration and/or the test duration in order to reduce the final algal biomass.

Test results should generally be expressed relative to measured exposure concentrations. Although analysis methods for cationic substances are incapable of distinguishing between bound and free test substance results should, nonetheless, be expressed in terms of measured exposures. For some regulatory applications it may be necessary to determine the extent to which the toxicity of a cationic substance is mitigated by adsorption to dissolved organic carbon. This topic is briefly addressed in Annex 3.

3.7 Complexing substances

Complexation may significantly affect the bio-availability and toxicity of a test substance. It may also reduce the availability in the test medium of salts (such as calcium and magnesium) and trace elements which are essential for supporting healthy test organisms. The following are examples of substances which may be involved in complexation:

- EDTA;
- Polyanionic polymers with carboxylic and phosphoric acids;
- Phosphonates; and
- Metals.

The extent to which a substance is complexed will depend upon the availability of co-complexing agents and other properties of the medium such as pH. Speciation models may be used to calculate the concentrations of dissolved and complexed test substance from the total nominal concentration added.

Data from tests in which complexation has been judged to have had a significant bearing on the result are likely to be of questionable value for classifying substances and for extrapolating to a predicted

no effect concentration for risk assessment. The extent to which effects are a direct consequence of chemical toxicity or a secondary effect, resulting from for example complexation induced nutrient limitation, should be determined where possible. Compensatory adjustment to water quality parameters or the testing of an appropriate salt of the test substance help to achieve a valid test result but protocols incorporating modifications to standard procedures should be validated and approved for use by the appropriate regulatory authority.

The effects of metal complexing substances in algal growth inhibition tests are mainly caused by chelation of essential cations, which leads to growth limiting reductions in the concentration of uncomplexed physiologically active ions. Inhibition of algal growth by metal complexing agents is therefore a secondary effect, which cannot be attributed to substance specific inherent toxic properties. The secondary effects can be eliminated by compensating for the deficit in the concentration of the essential ion(s). Guidance on toxicity mitigation testing with algae for chemicals which form complexes with and/or chelate polyvalent metals is given in Annex 4.

Analysis methods for quantifying exposure concentrations, which are capable of distinguishing between the complexed and non-complexed fractions of a test substance, may not always be available or economic. Where this is the case approval should be sought from the regulatory authority for expressing the test result in terms of nominal concentrations.

Complexation of metals to organic and inorganic ligands in test media and natural environments can be estimated from metal speciation models. Speciation models for metals, including pH, hardness, DOC, and inorganic substances such as MINTEQ (Brown and Allison, 1987), WHAM (Tipping, 1994) and CHESS (Santore and Driscoll, 1995) can be used to calculate the non-complexed and complexed fractions of the metal ions. Alternatively, the Biotic Ligand Model (BLM), allows for the calculation of the concentration of metal ion responsible for the toxic effect at the level of the organism. The BLM model has at present only been validated for a limited number of metals, organisms, and end-points (Santore and Di Toro, 1999). The models and formulae used for the characterisation of metal complexation in the media should always be clearly reported, allowing for their translation back to natural environments.

3.8 Coloured substances

The prime objective of aquatic toxicity tests is to determine the inherent toxicity of a substance. Coloured substances can present particular problems for determining true toxicity in algal tests and in tests with *Daphnia* sp. In fish tests, observation of behaviour and mortality of fish could also be difficult.

Algal tests

Coloured substances can absorb photosynthetically active light and hence limit growth of algal cultures. Absorption will be proportional to test substance concentration and as a consequence it can result in growth inhibition which is difficult to distinguish from inherent toxicity.

The need for clear guidance on how to determine the inherent toxicity of coloured substances to algae is widely recognised (EC, 1996a) and work is ongoing in this area (Justesen and Nyholm, 1998). The International Organization for Standardization (ISO, 1997) has identified the following general strategies for discriminating between effects resulting from toxicity and light absorption:

- testing at different light intensities, since the growth rate above light saturation is almost independent from the light intensity;
- reducing the light path by reducing the depth or the volume;

- 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; and
- utilising a light transmission filter by preparing reciprocal dilutions to the exposure concentrations thus maintaining a common light path length (constant depth) and the spectral absorbance characteristics of the sample.

Note: Care should be taken with the approaches described in the last two bullets. Utilising external filters is questionable because the light conditions in the test flasks are affected by a coloured substance in a much more complicated way than with the external absorption method. Instead, it is better to change the incubation system to compensate for the internal light absorption, thereby minimising its effect. In practice, this can be done by shortening the light path, increasing the light intensity, and maintaining a good turbulence (Justesen and Nyholm, 1998).

Specific methods under development which incorporate some of the above features have been described by Memmert (1994) and Comber *et al.*(1995). There is, however, limited experience of their application and as a consequence they cannot be endorsed at this stage.

Plant tests

Testing with the floating plant, *Lemna* sp., may provide a further alternative for coloured substances since it is unaffected by the optical properties of the test medium. A draft OECD Test Guideline for a growth inhibition test with this species has been developed but it still has to be established whether the test can be considered equivalent to the algal growth inhibition test.

Invertebrate tests

Observation of small invertebrate test organisms, such as *Daphnia* sp., can be difficult in highly coloured test media. Observations may be made easier by placing test vessels on a light box or transferring the contents of the test vessels to shallow containers for scoring.

3.9 Hydrophobic substances

Partitioning of test substances into or onto test organism biomass and onto food or other organic detritus in the test system is a potentially important loss mechanism for hydrophobic substances with high octanol/water partition coefficient (log K_{ow} >4) or bioconcentration factor (BCF >500). High octanol/water partition coefficients are generally associated with low water solubility and hence losses due to partitioning are likely to be more significant for hydrophobic substances.

Methods of preparing solutions of poorly water-soluble substances have been discussed in Section 3.1. Strategies for maintaining exposure concentrations of hydrophobic substances in aquatic toxicity test media include:

- reducing the ratio of test organism biomass to test medium volume (i.e. the use of a ratio of <1 g/l in fish tests);
- using a semi-static renewal or flow-through exposure regime;
- increasing the frequency or rate of test medium renewal in semi-static and flow-through tests;
- removing excess food and detritus;

- maintaining dissolved total organic carbon concentrations (other than that due to the test substance) in acute tests at or below 2 mg/l;
- feeding (e.g. of fish) a few hours before test medium renewal in semi-static tests; and
- making the system surfaces saturated with the compound so that it does not 'plate' out.

Analyses of exposure concentrations in algal tests with hydrophobic substances may need to take account of the potential for partitioning of the test substance onto the algal biomass. Where partitioning is likely to be significant it may be appropriate to quantify exposure following separation of algal cells from the test medium. Separation techniques include centrifugation and filtration and both of these are subject to the comments made in Section 3.1.1.

A film of hydrophobic test substance floating on the surface of test media may physically trap small aquatic invertebrates, such as daphnids. It should therefore either be removed before introducing the test organisms or the organisms should be prevented from coming into contact with it using screens, cages or other suitable devices. A device for preventing surface trapping of daphnids has been described by Dean and De Graeve (1986).

It is advisable to consult Section 3.6, since there is much overlap between hydrophobic substances and adsorbing substances.

3.10 Ionised substances

Relatively small changes in pH can significantly alter the balance between the dissociated and non-dissociated forms of some organic acids and bases. Altered dissociation equilibrium may in turn significantly affect the water solubility and partition coefficient of the substance, and thus also its bioavailability and measurable toxicity. It is, therefore, essential that the relevant dissociation constants (*pKa* values) are known prior to commencement of testing.

Design of the toxicity test should take into account the effects on dissociation equilibrium that adjustments to the pH may cause. A preliminary test, to determine the potential for differing toxicity of the two or more forms of the substance, should be considered where the *pKa* for the test substance falls within the normal pH range of a test. The definitive test should be conducted at a pH consistent with the more toxic form of the substance whilst remaining within the range required to maintain the health of the control organisms.

Where the substance itself causes a change to the pH of the test medium, the pH should be adjusted to lie within the specified range for the test using acid, alkali or other suitable buffer. It should be noted that the use of buffers can affect the result of the test - particularly for algae. Furthermore, this can cause sedimentation and/or degradation of the test substance. The suitability of any proposed buffer system should therefore be assessed prior to use in the definitive test. Adjustments to pH may be carried out in the stock solutions used to prepare the test media or in the media itself, as judged appropriate. In either case the procedure should be applied to all treatments, including the controls.

Growth of algal test cultures can cause increase of pH due to consumption of HCO_3 ions. Maintenance of stable pH when testing an ionised substance is therefore important to ensure that the balance between dissociated and non-dissociated forms of the substance is maintained. Strategies for maintaining the concentration of HCO_3 ions and therefore reducing pH shifts have been discussed in Section 3.4.

3.11 Multi-component substances

Mixtures comprising a complex mix of individual substances with different solubility and physical-chemical properties are frequently referred to as "complex mixtures". In most cases, they can be characterised as a homologous series of substances with a certain range of carbon chain length/number or degree of substitution (e.g. petroleum). In this Guidance Document, these are referred to as "multi-component substances". Different approaches to media preparation and/or testing are required for multi-component substances depending upon whether they are fully or partially soluble in the test media across a proposed range of test concentrations.

Fully soluble

Test methods consistent with those described for water-soluble substances are appropriate for mixtures comprising components which dissolve fully within the proposed range of test concentrations. Mixture components may, however, have individual properties (e.g. degradability, volatility etc.) that require steps to be taken to control losses.

Partially soluble

When multi-component substances is not fully soluble in water, it is important to seek to identify its components as far as practically possible and to examine the possibility of determining its toxicity using available information on its components. Only when the toxicity cannot be calculated, testing of the multi-component substances is considered.

The toxicity of complex multi-component substances, which are only partially soluble in water, can be determined by preparing water-accommodated fractions (WAFs) of them. The term water-accommodated fraction is applied to aqueous media containing only the fraction of multi-component substances that is dissolved and/or present as a stable dispersion or emulsion. Test data obtained with WAFs apply to the multi-component substances as an entity. Using the data derived from the testing of WAFs for classifying for aquatic toxicity is referred to in the draft Guidance Document on *Use of the Harmonized System for the Classification of Chemicals which are Hazardous for the Aquatic Environment* (OECD, 2000). It should be noted that applicability of WAFs depends on the objectives of regulatory authorities. When doing WAF testing, the appropriate regulatory authority should be consulted so that the data generated on WAFs are deemed appropriate by the regulatory authority receiving the data. For example, in the cases where poorly soluble components in the multi-component substances are of concern, the WAF method is not adequate to determine the toxicity of such multi-component substances.

WAFs are prepared individually and not by serial dilution of a single stock WAF. Measured amounts of 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. Following cessation of mixing and a period of settling (to allow phase separation) the aqueous phase, i.e. the WAF, is drawn off for testing. The duration of the mixing and settling phases should normally be determined by carrying out a preliminary study. Techniques such as turbidimetry and total organic carbon analysis may provide a useful indication of the progress of mixing and phase separation. Procedures for preparing WAFs of oil products and products such as creosote, have been described by Girling (1989) and Tadokoro *et al.* (1991) respectively.

WAFs should be prepared by adding carefully measured amounts of the substance to the test medium and then applying appropriate mixing techniques. The presence of water-miscible solvents can modify the composition of a WAF and as a consequence they should not be used during preparation. The severity of the mixing regime should be guided by the desire to attain an equilibrated test medium as

quickly as possible whilst avoiding, where possible, the formation of emulsions. Care should be taken due to the fact that some multi-component substances will have an innate tendency to form an emulsion or dispersion and will require to be tested as such (see Section 3.1.2 on "Dispersions and emulsions"). It is important to recognise that the duration of mixing and energy input can have a marked influence on the composition, particle size and proportion of dispersed and non-dispersed test material in the WAF. Generator systems described in Section 3.1.2 are not appropriate media preparation methods for multi-component substances. The method used to prepare the WAF should therefore be fully described in the test report and evidence of its compositional stability over time provided.

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. Similarly any fraction of the mixture forming a surface film should be decanted to prevent surface trapping. Where there is a need to retain an excess of the multi-component substances in the test vessels, steps should be taken to prevent trapping or fouling of test organisms (see Section 3.9 on "Hydrophobic substances").

Only a fraction of the total mass of multi-component substances responsible for the composition of a WAF may be present in the WAF. The "loading rate" has therefore been advocated for expressing exposures of mixtures that neither wholly dissolve nor completely form a stable dispersion or emulsion over the required test range (Girling *et al.*, 1992). The loading rate is the mass to volume ratio of the mixture to medium used in the preparation of a WAF. WAFs may be thus considered analogous to the term "nominal concentration" used for typical test substances, with all the limitations inherent to that term.

Chemical specific analysis is required to demonstrate attainment of equilibrium in WAF preparation and stability during the test. Methods capable of identifying gross changes in the composition of WAFs with time are required. Infra-red or ultra-violet spectroscopy or total peak area have been used successfully for this purpose. Total organic carbon analysis may also be useful in monitoring any losses of test material from test media but the low sensitivity of the method (approximately 1 mg/l) may limit its applicability.

Effects concentrations in tests based upon WAFs can be calculated from (1) the loading rates and are identified as either LL_{50} or EL_{50} values and/or (2) the measured mass of test substance in the WAF and are identified as either LC50 or EC50 values. For example, some regulatory authorities in the United-States require the latter approach so that an adequate risk assessment can be done for the test substance. LL_{50} or EL_{50} values are comparable to LC_{50} or EC_{50} values determined for pure substances tested within their solubility range. Similarly the NOEC (No Observable Effect Concentration) becomes the NOELR (No Observable Effect Loading Rate). The statistical methods used to determine LL_{50}, EL_{50} and NOELR values are the same as those used to determine LC_{50}, EC_{50} and NOEC values.

3.12 Preparations

Preparations are defined as a deliberate physical mixture of substances. Testing of preparations is only advocated in the following instances:

- where the toxicity of the preparation cannot be calculated from the toxicity of its component substances (N.B.: In this instance, consideration should first be given to determining the toxicity for any of the component substances for which data are lacking, so that the toxicity of the preparation can then be calculated);
- where data confirming the calculated toxicity are specifically requested; and
- where there is a regulatory requirement which is commonly the case for registration of pesticides and biocides.

Where testing of the preparation as a whole is required, the approaches identified above for multi-component substances should be considered.

In general, alloys can be considered separately from other preparations due to their unique physical and chemical properties that differentiate them from simple mixtures of constituent elements. An alloy can be defined as a metallic material, homogeneous on a macroscopic scale, consisting of two or more elements so combined that they cannot be readily separated by mechanical means. Due to the decreased solubility or lack of solubility of many alloys, the approaches identified for multi-component substances are not appropriate. However, care should be taken due to the fact that some alloys such as powered brass show toxicity to aquatic organisms.

4. Sampling of test media for analysis of exposure concentrations

A detailed consideration of analysis techniques for use in aquatic toxicity tests is outside the scope of this document. Sections 4.1 and 4.2 are therefore limited to providing guidance on the design of sampling schedules and methods of sampling.

4.1 Sampling schedules for analysis of test media

Schedules for collecting samples of test media for analysis are often included in testing guidelines. The following recommendations are designed to supplement the information given in the guidelines and provide a lead where guidance is lacking.

Sampling schedules for analytical support to tests with algae, plants, daphnids and fish are outlined below. These schedules only apply to tests carried out within the solubility range of pure substances. It should be noted that for difficult substances there is a risk in analysing only the minimum number of samples that the exposure concentrations will be inadequately described. As a general rule it is therefore advisable, to schedule for sampling all concentrations at a higher frequency. Under some circumstances it may be possible to take additional samples and preserve them using fully validated methods. Analysis of the additional samples is only carried out if results from the minimum sample set provide insufficient data to adequately quantify exposure. It is recommended that mean measured concentrations be taken at the beginning of the test, and 24-hour intervals throughout the test in order to obtain the mean measured concentrations, where losses due to e.g. volatility are anticipated. It should also be noted that where the flow-through exposure system is selected, analytical samples should be taken at the beginning of the test to verify that the system is stable and operating correctly.

4.1.1 Algal growth inhibition test

Test media prepared specifically for analysis of exposure concentrations should be treated the same as those used for testing i.e. they should be inoculated with algae and incubated under test conditions. It may be necessary to subsequently separate algae from the medium or to analyse a series of test solutions incubated under test conditions but with no algae in order to obtain a correct determination of the dissolved test substance concentration (see Section 3.9). Since requirements may vary between different regulatory authorities, it is good practice to consult with the appropriate authority.

Analysis at the start and end of the test of a low and high test concentration and a concentration around the expected EC_{50} may be sufficient where it is likely that exposure concentrations will remain within 20% of nominal values. Analysis of all test concentrations at the start and end of the test is required where concentrations are unlikely to remain within 80-120% of nominal.

4.1.2 *Lemna* growth inhibition test

This test may be carried out using static, semi-static renewal or continuous flow-through exposure regimes.

Static

A static test is appropriate where exposure concentrations are expected to remain within 80-120% of nominal over the 7-day exposure period. Analysis of the highest and lowest test concentration and a concentration around the expected LC_{50} at the start and end of the exposure period is considered the minimum requirement.

Semi-static

A semi-static renewal regime is recommended where exposure can not be maintained within 80-120% of the nominal values over the test duration (7 days). Analysis of the highest and lowest test concentrations and a concentration around the expected LC_{50} at the start and end of each renewal period is recommended. Renewing the test media on, at least, two occasions during the test (e.g. days 3 and 5), is considered the minimum requirement.

Continuous flow-through

A continuous flow-through regime may be preferred in some circumstances, e.g. where concentrations are expected to decline from nominal by more than 20% over 24 hours.

If, under flow-through conditions, measured concentrations are likely to remain within 80-120% of nominal then the highest and lowest test concentrations and a concentration around the expected LC_{50} should be analysed at the start, mid-way through and at the end of the test. If the concentrations are expected to decline by more than 20% then all the concentrations should be analysed at the start, mid-way through and at the end of the test.

4.1.3 *Daphnia* acute immobilisation test

Analysis of highest and lowest test concentrations and a concentration around the expected EC_{50} at the start and end (typically after 48 hours) of the exposure period may be sufficient where it is likely that exposure concentrations will remain within 80-120% of nominal values. Analysis of all test concentrations is required where concentrations are unlikely to remain within 80-120% of nominal.

Daphnia acute immobilisation test might be carried out using semi-static renewal or flow-through regimes, but these approaches are not prevalent due to practical and cost reasons. Renewal systems are not recommended in the OECD Test Guideline.

4.1.4 *Daphnia* reproduction test

This test may be carried out using semi-static renewal or continuous flow-through exposure regimes.

Semi-static

Where the concentration of the test substance is expected to remain within 80-120% of nominal, it is recommended that the highest and lowest concentrations and a concentration around the expected NOEC/EC_x are analysed when freshly prepared and immediately prior to renewal on one occasion during the first week and thereafter weekly during the test. Where the test concentrations are not expected to remain within 80-120% of nominal, it is necessary to either analyse all concentrations or consider a continuous flow- through test.

Continuous flow-through

Where the test substance concentrations are expected to remain within 80-120% of nominal, it is recommended that the highest and lowest test concentrations and a concentration around the expected NOEC/EC_x are analysed three times during the first week and thereafter weekly during the test. Where the test concentrations are not expected to remain within 80-120% of nominal, all the concentrations should be analysed three times during the first week and thereafter weekly during the test.

4.1.5 Fish acute toxicity test

The test may be carried out using static, semi-static renewal or continuous flow-through exposure regimes.

Static

A static test is appropriate where exposure concentrations are expected to remain within 80-120% of nominal over the 96 hour exposure period. Analysis of the highest and lowest test concentration and a concentration around the expected LC_{50} at the start and end of the exposure period is considered the minimum requirement.

Semi-static

A semi-static renewal regime is recommended where exposure can be maintained within 80-120% of the nominal values by renewing the test media at 24 or 48 hour intervals. Analysis of the highest and lowest test concentrations and a concentration around the expected LC_{50} at the start and end of the first 24 or 48 hour renewal period is considered the minimum requirement.

Continuous flow-through

A continuous flow-through regime is recommended where concentrations are expected to decline from nominal by more than 20% over 24 hours.

If, under flow-through conditions, measured concentrations are likely to remain within 80-120% of nominal then the highest and lowest test concentrations and a concentration around the expected LC_{50} should be analysed at the start, mid-way through and at the end of the test. If the concentrations are expected to decline by more than 20% then all the concentrations should be analysed at the start, mid-way through and at the end of the test.

4.1.6 Fish prolonged toxicity test, fish early-life stage toxicity test, fish juvenile growth toxicity and fish short-term toxicity on embryo and sac-fry stages

Fish prolonged toxicity test, fish early-life stage toxicity test, fish juvenile growth toxicity and fish short-term toxicity on embryo and sac-fry stages are classified into this category. These tests may be carried out using semi-static renewal or continuous flow-through exposure regimes.

Semi-static

A semi-static renewal regime is recommended where exposure can be kept to within 80-120% of the nominal values by renewing the test media at intervals. Analysis of the highest and lowest test concentrations and a concentration around the expected NOEC/EC_x at the start and end of the first renewal period and thereafter at least weekly is considered the minimum requirement.

Continuous flow-through

A continuous flow-through regime is recommended where concentrations are expected to decline from nominal by more than 20% over 24 hours.

If, under flow-through conditions, measured concentrations are likely to remain within 80-120% of nominal then the highest and lowest test concentrations and a concentration around the expected NOEC/EC_x should be analysed three times during the first week and thereafter at least at weekly intervals. If the concentrations are expected to decline by more than 20% then all the concentrations should be analysed three times during the first week and thereafter at least at weekly intervals.

4.2 Taking of media samples for chemical analysis

Sampling of test media for chemical analysis will be case specific and it is therefore not possible to give guidance which will be applicable in all cases. However, it is likely that it will be important to consider the following when developing a suitable method:

- Does the test substance have physical-chemical properties which warrant special consideration. The properties of difficult test substances discussed earlier in the context of testing may also be relevant to sampling and analysis?
- What sample volume is required in order to measure exposure to the required level of accuracy and precision?
- Will it be necessary to set up additional test vessels in order to obtain an adequate sample volume?
- What method of sampling should be employed and where in the test vessels should samples be taken?
- What time interval is acceptable between taking and analysing samples?
- Do samples require immediate fixing or extraction into an organic solvent?
- Is sample storage acceptable and if so are there special requirements?

Sample collection and storage methods should be validated before they are applied to a definitive test.

5. Calculation and expression of test results

The procedures described in Section 4 provide either nominal or measured exposure concentrations of pure substances which can be used to determine effects concentrations. The following general principles apply with respect to how these concentrations are to be used when calculating effect concentrations in accordance with test guidelines:

- For static and semi-static and flow-through tests, where the concentrations remain within 80-120% of nominal, the effect concentrations can be expressed relative to nominal or measured concentrations;
- For static and semi-static tests, where the concentrations do not remain within 80-120% of nominal, the effect concentrations could be determined and expressed relative to the geometric mean of the measured concentrations. A formula for calculating the geometric mean is given in Annex 2;
- For flow-through tests, where the concentrations do not remain within 80-120% of nominal, the effects concentrations should be determined and expressed relative to the arithmetic mean concentration; and
- For tests with chemicals that cannot be quantified by analytical methods at the concentrations causing effects, the effect concentration can be expressed based on the nominal concentrations.

It should be noted that several types of loss processes can occur very fast and new equilibrium conditions can be attained relatively quickly during the exposure. If this is the case, the median of the concentrations which are measured after the decline would be more appropriate as a surrogate for the mean exposure concentration. Since there may be various methods for determining that, the method to determine mean measured concentrations should be made explicit in the reporting of test results (see Section 3.3).

It is generally recommended that all test results be expressed in terms of mean measured concentrations as far as possible. Some regulatory authorities invalidate, in certain regulatory context, toxicity data from tests conducted above the aqueous water solubility limits using nominal concentrations. It should also be noted that it is often useful to have both measured and nominal effect concentrations quoted.

Guidance on the conventions used for expressing exposure and determining effect concentrations in tests with multi-component substances is given in Section 3.11.

6. References

ASTM (1997). Standard guide for conducting *Daphnia magna* life cycle toxicity tests. American Society for Testing and Materials, West Conshohocken, PA, Guideline E-1193-97.

Auer, C.M., Nabholz, J.V., and Baetcke, K.P. (1990), Mode of Action and the Assessment of Chemical Hazards in the Presence of Limited Data: Use of Structure-Activity Relationships (SAR) under the US Toxic Substances Control Act, Section 5. *Environ. Health Perspectives*, **87**, 183-197.

Benville, Jr., P.E. and Korn, S. (1974). A simple apparatus for metering volatile liquids into water. *J. Fish. Res. Board. Canada,* **31(3)**, 367-368.

Billington, J.W. (1988). Preparation of aqueous solutions of sparingly soluble organic substances: 1. Single component systems. *Environ. Toxicol. Chem.* **7**, 117-124.

Boethling, R.S. and Nabholz, J.V. (1997). Environmental assessment of polymers under the U.S. Toxic Substances Control Act, Chapter 10. pp 187-234 in Hamilton, J.D. and Sutcliffe, R. (editors), Ecological Assessment of Polymers: Strategies for Product Stewardship and Regulatory Programs. Van Nostrand Reinhold, New York. 345 p.

Brown, D.S. and Allison, J.D. (1987). MINTEQA1 Equilibrium Metal Speciation Model: A user's manual. Athens, Georgia, USEPA Environmental Research Labaoratory, Office of Research and Development.

Comber, M.H.I., Smyth, D.V. and Thompson, R.S. (1995). Assessment of the toxicity to algae of coloured substances. *Bull. Environ. Contam. Toxicol.*, **55**, 922-928.

CONCAWE - The Oil companies European Organisation for Environment, Health and Safety. (1992). Ecotoxicological testing of petroleum products: test methodology. Report No. 92/56, CONCAWE, Brussels.

Dean, J.H. and De Graeve, G.M. (1986). A screen device to eliminate "floaters" in *Daphnia magna* toxicity tests. *Environ. Toxicol. Chem.*, **5**, 1055-1057.

Diamantino, T.C., Ribeiro, R., Gonzalves, F. and Soares, A.M.V. (1997). METIER (Molecular Ecotoxicity Tests Incorporating Ecological Relevance) for difficult substances. 4: Test chambers for cladocerans in flow-through conditions. *Environ. Toxicol. Chem.*, **16**(6), 1234-1238.

Department of Environment in the United-Kingdom. (1996). Guidance on the aquatic toxicity testing of difficult substances. Published in May 1996 by the United Kingdom Department of the Environment, London.

European Commission (1996a). Ad-hoc meeting of experts on algal growth inhibition test for coloured substances, Vienna, 30-31 January 1996. Report of the Working Group: Final Draft, 4 April 1996. European Chemicals Bureau, Joint Research Centre, Ispra, Italy.

European Commission (1996b). Technical Guidance Document in support of Commission Directive 96/67/EEC on risk assessment of new notified substances and Regulation (EC) No. 1488/94 on risk assessment of existing substances Parts I, II, III and IV). EC catalogue numbers CR-48-96-001, 002, 003, 004-EN-C. Office for Official Publications of the European Community, 2 rue Mercier, L-2965 Luxembourg.

ECETOC - The European Centre for Ecotoxicology and Toxicology of Chemicals. (1996). Aquatic toxicity testing of sparingly soluble, volatile and unstable substances. ECETOC Monograph No 26, Brussels.

Gingerich, W.H., Seim, W.K. and Schonbrod, R.D. (1979). An apparatus for the continuous generation of stock solutions of hydrophobic chemicals. *Bull. Environ. Contam. Toxicol.* **23**, 685-689.

Girling, A.E. (1989). Preparation of aqueous media for aquatic toxicity testing of oils and oil-based products: a review of the published literature. *Chemosphere*, **19**(10/11), 1635-1641.

Girling, A.E., Markarian, R.K. and Bennett, D. (1992). Aquatic toxicity testing of oil products - some recommendations. *Chemosphere*, **24**(10), 1469-1472.

Halling-Soerensen, B., Nyholm, N. and Baun, A. (1996). Algal-toxicity tests with volatile and hazardous compounds in air-tight test flasks with CO_2 enriched headspace. *Chemosphere*, **32**, 1513-1526.

ISO - International Organization for Standardization (1997). Water quality - Sampling - Part 16: Guidance on biotesting of samples. International Standard ISO 5667/16.

ISO - International Organization for Standardization (1998). Water quality - Guidance for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water. International Standard ISO 14442.

Justesen, K. and Nyholm, N. (1998). Protocol for algal toxicity test with coloured substances. - Initial study for the Commission of the European Communities. Contract No 13781-1998-03 F1ED ISP DK.

Lynch, D.G., Macek, G.J., Nabholz, J.V., Sherlock, S.M. and Wright, R. (1994). Ecological Risk Assessment Case Study: Assessing the Ecological Risks of a New Chemical Under the Toxic Substances Control Act. Pages 1-1 to 1-B4 (Section One) in: A Review of Ecological Assessment Case Studies From A Risk Assessment Perspective, Volume II. Washington, DC: Risk Assessment Forum, Office of Research and Development, U.S. Environmental Protection Agency, Report No. EPA/630/R-94/003.

Mackay, D. (1992). Multimedia Environmental Models, Lewis Publishers.

Mayer, P.; Nyholm, N., Verbrüggen , E. M. J. , Hermens, J.L.M., Tolls, J. (2000).: An algal growth inhibition test in filled, closed bottles for volatile and sorptive materials, Environmental Toxicology and Chemistry (in press).

Memmert, U. (1994). Inhibition of algal growth caused by coloured test substances. RCC Interim Report Project 460302, RCC Ltd., Switzerland.

Mount, D.I. and Brungs, W.A. (1967). A simplified dosing apparatus for fish toxicology studies. *Water Res.*, **1**, 21-30.

Nabholz, J. V. (1991). Environmental hazard and risk assessment under the United States Toxic Substances Control Act. *The Science of the Total Environment*, **109/110**, 949-665.

Nabholz, J. V., Miller, P. and Zeeman, M. (1993) "Environmental Risk Assessment of New Chemicals Under the Toxic Substances Control Act (TSCA) Section 5: "Environmental Toxicology and Risk Assessment, ASTM STP 1179, Wayne G. Landis, Jane S. Hughes, and Michael. A. Lewis, Editors. American Society for Testing and Materials, Philadelphia, 1993, 40-55.

Newsome, L.D., Nabholz, J.V. and Kim, A. (1996). Designing Aquatically Safer Chemicals, Chapter 9. Pages 172-192 in: DeVito, S.C. and Garrett, R.L. (Editors). Designing Safer Chemicals: Green Chemistry for Pollution Prevention. ACS Symposium Series No. 640. American Chemical Society, Washington, DC. 264p.

OECD (1995). Draft (November 1995) report of the OECD Workshop on Aquatic Toxicity Testing of Sparingly Soluble Metals, Inorganic Metal Compounds and Minerals. Ottawa, Canada, September 5-8, 1995.

OECD (2000). Draft Guidance Document on the Use of the Harmonized System for the Classification of Chemicals which are Hazardous for the Aquatic Environment (April 2000), OECD, Paris.

Phipps, G.L., Holcombe, G.W. and Fiandt, J.T. (1982). Saturator system for generating toxic water solutions for aquatic bioassays. *Progressive Fish Culturist*. **44**(2), 115-116.

Santore, R.C. and Driscoll, C.T. (1995). The CHESS Model for Calculating Chemical Equilibria in Soils and Solutions, Chemical Equilibrium and Reaction Models. The Soil Society of America, American Society of Agronomy.

Santore, R.C. and Di Toro, D.M. et al (1999). A biotic ligand model of the acute toxicity of metals. II. Application to fish and daphnia exposure to copper. *Environ. Toxicol. Chem.* Submitted.

Sousa, J.V., McNamara, P.C., Putt, A.E., Machado, M.W., Suprenant, D.C., Hamelink, J.L., Kent, D.J., Silberhorn, E.M. and Hobson, J.F. (1995). Effects of octamethylcyclotetrasiloxane (OMCTS) on freshwater and marine organisms. *Environ. Toxicol. Chem.*, **14**(10), 1639-1647.

Tadokoro, H., Maeda, M., Kawashima, Y., Kitano, M., Hwang, D. and Yoshida, T. (1991). Aquatic toxicity testing for multicomponent compounds with special reference to preparation of test solutions. *Ecotox. Environ. Safety.*, **21**, 57-67.

Tarr, A.L. and Hutchinson, T.H. (1992). A review of the use of solvents in aquatic toxicity testing. ICI Group Environmental Laboratory Report BL 4443/A.

Tipping, E. (1994). WHAM – A computer equilibrium model and computer code for waters, sediments, and soils incorporating discrete site/electrostatic model of ion-binding by humic substances. Computers and Geoscience 20 (6): 073-1023.

Tjeerdema, R.S. and Singer, M.M. (1991). Closed flow-through aquatic toxicity testing and microscopic organisms: not necessarily incompatible. *Mar. Poll. Bull.* **22**(2), 59-61.

Tolls, J., Haller, M., de Graaf, I., Thijssen, M.A.T.C. and Sijm, D.T.H.M. (1997). Bioconcentration of LAS. Experimental determination and extrapolation to environmental mixtures. *Environ. Sci. Technol.* **31**, 3426-3431.

Urrestarazu Ramos, E, Vaes, W.H.J., Verharr, H.J.M. and Hermens, J.L.M. (1997). A novel approach for dissolving chemicals with low aqueous solubility: generator disk in the headspace. *Environ. Toxicol. Chem.*, **16**(11), 2229-2231.

US EPA (1996a). Ecological effects test guidelines - OPPTS 850.1000: Special considerations for conducting aquatic laboratory studies. Public Draft, EPA 712-C-96-113, United States Environmental Protection Agency. Available from http://www.epa.gov/opptsfrs/OPPTS_Harmonized.

US EPA (1996b). EPA Ecological effects test guidelines - OPPTS 850.1300: Daphnid Chronic Toxicity Test. Public Draft, EPA 712-C-96-120, United States Enviromental Protection Agency. Available from http://www.epa.gov/docs/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/.

Van Leeuwen, C.J.A., Espeldoorn, A. and Mol, F. (1986). Aquatic toxicological aspects of dithiocarbamates and related compounds. Embryo-larval studies with rainbow trout (*Salmo gairdneri*). *Aquatic Tox.*, **9**, 129-145.

Veith, G.D. and Comstock, Y.M. (1975). Apparatus for continuously saturating water with hydrophobic organic chemicals. *J. Fish. Res. Board Can.* **32**, 1849-1851.

VKI (1998). Handling of difficult organic substances in aquatic toxicity tests. Report to the Danish EPA, September, 1998. VKI, Denmark.

Annex 1

Computer programs for predicting physical/chemical properties, and fate, transport and aquatic toxicity of substances

The following computer programs are used by the Office of Pollution Prevention and Toxics (OPPT) of the United States Environmental Protection Agency for estimating physical/chemical properties, and fate, transport and toxicity of substances.

1. Physical/chemical properties, fate and transport.

"EPI" (Estimation Programs Interface)

Available from:

Syracuse Research Corp, 6225 Running Ridge Rd, N. Syracuse, NY 13210, U.S.A., Tel: 315-452-3350, Fax: 315-426-3429, Email: howardp@syrres.com, Internet: http://esc.syrres.com.

and

Peter Fisk Associates, 9 St. Swithins Road, Tankerton, Whitstable, Kent, CT5 2HT, U.K., Tel: 01227 273797, Fax: 01227 794982, Email: pfa@enterprise.net, Internet: http://www.ecotoxchem.co.uk

2. Aquatic toxicity

"ECOSAR"

Available as a computer program with a user's manual and a technical support manual from:

J. V. Nabholz, RAD(7403), USEPA, 401 M St, SW, Washington, DC 20460-0001, U.S.A., Tel: 202-260-1271, Email: nabholz.joe@epa.gov, Internet: http://www.epa.gov/opptintr/newchms/

and

P. H. Howard, Syracuse Research Corp. 6225 Running Ridge Rd, N. Syracuse, NY 13210, U.S.A., Tel: 315-452-8417, Email: howardp@syrres.com).

and

Peter Fisk Associates, 9 St. Swithins Road, Tankerton, Whitstable, Kent, CT5 2HT, U.K., Tel: 01227 273797, Fax: 01227 794982, Email: pfa@enterprise.net, Internet: http://www.ecotoxchem.co.uk

or as a manual - Clements, R.G. (editor), Nabholz, J.V., and Zeeman, M. (1996). Estimating toxicity of industrial chemicals to aquatic organisms using structure activity relationships. 2nd edition. 402 p.

from:

New Chemicals Screening Branch, Risk Assessment Division (7403), Office of Pollution Prevention and Toxics, United States Environmental Protection Agency. EPA Report No. EPA-748-R-93-001. Washington, DC 20460-0001.

3. n-Octanol-water partition coefficient (log K_{ow})

"ClogP"

Available from:

BioByte Corporation, 201 W. Fourth St., Suite 204, Claremont, CA 91711-4707, U.S.A., Tel: 909-624-5992, Fax: 909-624-1398, Email: clogp@biobyte.com, Internet: http://www.biobyte.com/~clogp/

"KOWWIN"

Available from:

Syracuse Research Corporation Corp, 6225 Running Ridge Rd, N. Syracuse, NY 13210, U.S.A., Tel: 315-452-3350, Fax: 315-426-3429, Email: howardp@syrres.com, Internet: http://esc.syrres.com.

and

Peter Fisk Associates, 9 St. Swithins Road, Tankerton, Whitstable, Kent, CT5 2HT, U.K., Tel: 01227 273797, Fax: 01227 794982, Email: pfa@enterprise.net, Internet: http://www.ecotoxchem.co.uk

"AUTOLOGP"

Described by:

Devillers, J., Domine, D. and Karcher, W. (1995). SAR QSAR. *Environ. Res.*, **3**, 301-306.

Annex 2

Formula for calculating geometric mean exposure concentration

The following formula allows calculation of a geometric mean exposure concentration where concentrations have been determined on more than two occasions during a test.

$$\text{Mean concentration} = \text{antilog}\left(\frac{1}{2(t_n - t_1)}\sum_{i=1}^{n-1}\left[\left(\log(conc_i) + \log(conc_{i+1})\right)\cdot(t_{i+1} - t_i)\right]\right)$$

Where: t_1 = initial time < t_2 <….. t_n = final time
$conc_1$ = initial concentration, $conc_2$, ….., $conc_n$ = final concentration

Annex 3

Toxicity mitigation testing for cationic substances

Under some circumstances it may be required to determine the extent to which the toxicity of a substance is mitigated by adsorption to dissolved organic carbon. For example, where cationic substances show moderate to high aquatic toxicity in dilution water containing <2 mg/l total organic carbon. Toxicity mitigation testing is usually carried out with fish. Fish are least susceptible to physical effects resulting from clogging and/or coating of gills by precipitate formed by the reaction of the cationic substance with the anionically charged dissolved organic carbon. Mitigation testing has, however, also been successfully carried out with daphnids and green algae.

Once the intrinsic toxicity of a cationic substance has been determined in dilution water containing less than 2.0 mg/l total organic carbon, the substance should be tested at least two more times with two different concentrations of dissolved humic acid. The first of these tests should be carried out at a humic acid concentration of 20 mg /l (or lower if a floc, precipitate, or a viscous mixture forms). The second test should be carried out with a reduced humic acid concentration, for example, 10 mg/l.

The concentration of total organic carbon should be measured in the controls of the test without added humic acid, the test with 20 mg/l humic acid and the test with 10 mg/l humic acid. TOC samples should be taken from the controls at the beginning of the toxicity tests and it is recommended that total organic carbon be measured three times in each of the three tests. The three measurements should be reported separately and with the mean value for each test.

Toxicity mitigation is determined by regressing the effect concentrations determined in the test (EC_{50}, LC_{50} values etc.) against the total organic carbon concentration.

Annex 4

Toxicity mitigation testing with algae for chemicals which form complexes with and/or chelate polyvalent metals

The requirements for algal toxicity testing in relation to risk assessment for substances which complex metals depend upon the water quality which is envisaged in the release scenario. The following testing scheme has been suggested by the US EPA:

1. test chemical as it is in standard algal growth medium with a hardness in the range 15 to 24 mg/l as $CaCO_3$;
2. test chemical as it is in modified algal growth medium with a hardness of approximately 150 mg/l as $CaCO_3$;
3. test the chemical as the Ca salt in standard algal medium by adding an equivalent amount of Ca^{2+} to the stock solution; and
4. test the chemical as the Ca salt in modified algal medium as for (2) above.

Testing a substance as the Ca salt requires the addition of an equivalent of Ca^{2+} to the stock solution. A suitable procedure for preparing a calcium salt of a substance might be to add 1 g active ingredient of the substance to a 1 litre volumetric flask which is partly filled with water and being stirred continuously. An equivalent of Ca^{2+} is then added and stirred for at least one hour. The flask is then topped up to 1 litre with water and used to prepare test media. Precipitate and/or flocculant which forms in the stock solution should be maintained, to the extent possible, as a homogeneous dispersion during preparation of the test media and should not be removed by filtration or centrifugation.

Annex 5

Expert Panel on Aquatic Toxicity Testing of Difficult Substances

Peer Review Committee

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