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GUIDANCE DOCUMENT ON AQUEOUS-PHASE AQUATIC TOXICITY TESTING OF DIFFICULT TEST CHEMICALS

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No. 23 (Second Edition)

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Series on Testing and Assessment

No. 23

GUIDANCE DOCUMENT ON AQUEOUS-PHASE AQUATIC TOXICITY TESTING OF DIFFICULT TEST CHEMICALS

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2018

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Foreword

This document provides the second edition of Guidance Document (GD) 23, on Aqueous-Phase Aquatic Toxicity Testing of Difficult Test Chemicals.

The project to develop this Guidance Document was co-led by the European Commission (EC-JRC) and the United States, assisted by the International Council for Animal Protection in OECD Programmes (ICAPO).

The first edition of GD 23, on Aquatic Toxicity Testing of Difficult Substances and Mixtures, was published in 2000 and required revision due to significant advances in the methods available for the conduct of aquatic toxicity tests with difficult to test substances.

The Second Edition of the Guidance Document was approved by the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) at its 30th meeting in April 2018. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 30 June 2018.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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1. GLOSSARY OF IMPORTANT TERMS USED IN THE GUIDANCE DOCUMENT

All terms and their descriptions should be considered as working definitions for the purpose of this Guidance Document only.

Chemical dispersant / emulsifying agent	A chemical reagent (e.g. surfactant) which aids in the production of a suspension / emulsion of a test chemical.
Critical micelle concentration	A maximum concentration of the freely solubilised surfactant in water.
Emulsion	A dispersion of liquid droplets in another liquid. (Note: both liquids are immiscible.)
Impurity	Any component of the test chemical that is not the desired entity.
Liquid-liquid saturator	A means to generate a saturated solution of a poorly soluble liquid test chemical using a vessel, such as a carboy, where the liquid test chemical is in direct contact with an aqueous phase.
Loading rate	The ratio of test chemical to test medium (in mg/L) used in the preparation of a WAF, a saturated solution, dispersion of a poorly water soluble test chemical (dosed above water solubility), or test solution. For unstable test chemicals, the loading rate refers to the parent test chemical.
Mixture/Preparation	A deliberate physical mixture of test chemicals that do not produce a substance that is different from the substances that were combined, including a prepared formulation or hydrate. Mixtures or preparations are generally fully characterised in terms of their components.
Mono-component substance	Test chemical which consist of a single predominant chemical component with the remaining minor components of impurities.
Multi-component substance (MS)	Test chemical, including UVCBs or mixtures/preparations comprised of a mix of two or more individual chemical components.
Passive dosing	Refers to using a polymer as a loaded compartment to achieve a target equilibrium concentration of the test chemical in the aqueous phase at or below saturation.
Poorly/sparingly water-soluble test chemical	Test chemical with a limit of water solubility of <100 mg/L.

Saturation concentration	The maximum dissolved concentration of a test chemical that can be achieved under the test conditions.
Saturator column	A means to generate a saturated solution of a poorly soluble test chemical by passing dilution water through a column loaded with an inactive high surface area matrix (e.g. glass beads / glass wool) coated with the test chemical.
Solid-liquid saturator	A means to generate a saturated solution of a poorly soluble solid test chemical using the inside surface of a glass carboy or vessel as an area, which when coated with the test chemical, generates a stock solution up to saturation.
Stable exposure concentration	A condition in which the exposure concentration remains within 80-120% of nominal or mean measured values over the entire exposure period.
Stock solution	A solution at a higher concentration used to facilitate achieving a target concentration.
Surfactant	A surface active agent that lowers the surface tension of the liquid in which it is dissolved.
Suspension	A dispersion of solid particles in a liquid.
Test chemical	What is being tested.
Test medium	Aqueous solution that constitutes the environment of the exposure system, but without test chemical or solvent.
Test solution	Test medium with the test chemical(s) at the target test concentration (may also include solvent).
Unknown or Variable Composition, Complex reaction products and Biological materials (UVCB)	UVCBs are comprised of a complex mix of individual components. These test chemicals may not have a precise chemical name, and they usually do not have a complete chemical structure diagram or a specific molecular formula associated with the test chemical. Therefore, a broader indication of the nature of the test chemical is given instead (e.g. identity of starting materials, carbon range, or genus/species information if plant derived).
Unstable exposure concentration	A condition in which the exposure concentration does not remain within 80-120% of nominal or mean measured values over the entire exposure period.
Water-accommodated fraction (WAF)	An aqueous fraction containing the dissolved and/or suspended and/or emulsified fraction of an MS.

Water-miscible solvent	A solvent in which a test chemical can be dissolved to facilitate preparation of stock solutions and/or test solutions.
Water solubility	The maximum attainable concentration or the concentration at thermodynamic equilibrium between an aqueous phase (deionised or distilled water) and a solid, liquid, or gaseous pure phase determined under standardised conditions, e.g. OECD TG 105. For MSs water solubility refers to the properties of the individual components.
Water soluble fraction	The result of a WAF that is subjected to a separation step (e.g. centrifuged or filtered through suitable filters) to remove any suspended undissolved emulsified components.

2. ABBREVIATION LIST

BCF	Bioconcentration factor
CMC	Critical micelle concentration
DOC	Dissolved organic carbon
EC_{50}	Median effective concentration
EL_{50}	Median effective loading
GC	Gas chromatography
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography-mass spectrometry
IR spectroscopy	Infra-red spectroscopy
LC_{50}	Median lethal concentration
LogD	Logarithmic octanol-water distribution constant at a specific pH
$LogP_{ow}$	Logarithmic octanol-water partition constant
LL_{50}	Median lethal loading
NOEC	No-observed effect concentration
NOELR	No-observable effect loading rate
MS	Multi-component substance
PDMS	Polydimethylsiloxane
pK_a	Acid dissociation constant
TG	Test guideline
TOC	Total organic carbon
(Q)SAR	(Quantitative) structure-activity relationship
UVCB	Unknown or Variable composition, Complex reaction products and Biological materials
UV/VIS spectroscopy	Ultra-violet/visible spectroscopy
WAF	Water-accommodated fraction

3. INTRODUCTION

1. Guidance for the aquatic toxicity testing and assessment of difficult test chemicals was identified as a high priority requirement by the National Coordinators of the Test Guidelines Programme and the Risk Assessment Advisory Body of the OECD, which resulted in the development of the first Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures and its publication in 2000 as number 23 in the OECD Series on Testing and Assessment.

2. The first guidance document was prepared following a meeting of the Expert Panel on the 2-3 April 1998 in Paris, France, where relevant documents published by the United Kingdom Department of the Environment, the United States Environmental Protection Agency, the International Organization for Standardization, the European Centre for Ecotoxicology and Toxicology of Chemicals, the Oil Companies European Organisation for Environment, Health and Safety and the Danish Water Quality Institute were considered.

3. In 2014 a project proposal was submitted to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) to partly revise the Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Subsequent WNT-evaluation in 2015 resulted in an adapted work plan for this proposal and full revision of Guidance Document 23.

4. The draft revised Guidance Document was circulated to national experts through the OECD National Co-ordinators of the Test Guidelines Programme for additional commenting in 2017 and was discussed during meetings of the OECD Validation Management Group on Ecotoxicity (VMG-Eco) from 2016-2017.

4. SCOPE

5. This document provides guidance for the testing of test chemicals, classed as "difficult to test" for the purposes of determining their aquatic toxicity. This guidance is applicable to aquatic tests of the dissolved fraction of test chemicals. The dissolved fraction of a test chemical is considered most relevant because effect relationships observed in aquatic toxicity tests are generally best explained when considered in relation to exposure concentrations of the dissolved test chemical. Consistent with the guidance presented herein, this document is generally not applicable to aquatic tests which include the undissolved phase of a test chemical, except where noted in this guidance for test chemicals which form stable dispersions. It should be noted that specific OECD guidance documents are under development for aquatic toxicity tests of test chemicals considered as nanomaterials, which may include both dissolved and undissolved fractions in the exposure.

6. OECD Guidelines for which this guidance is applicable include those that involve dissolved test chemical in aqueous solution in OECD Series 100, 200, 300, and 400. It should be noted that this guidance does not replace test guidelines and is meant to supplement the test guidelines when more specific information is not provided for testing of difficult test chemicals. This guidance does not address testing of biological pesticides that are living organisms.

7. The guidance relates to the practical aspects of carrying out valid tests with "difficult" test chemicals and presenting the results. As such it considers those test chemicals with the properties described in Table 1 that require modifications or additions to standard testing procedures. Guidance herein does not extend to interpretation of results, after testing of these test chemicals, or their use in classification and risk assessment.

8. The guidance is considered to reflect good practices, but methods and approaches for the assessment of "difficult" test chemicals are evolving and, as such, best practices may change as well. Where possible, the experimental set-ups described in this guidance should be approved by the relevant regulatory authority. It is also important to recognise that some test chemicals will present specific scientific and technical issues that may fall outside the scope of this guidance. In addition, it is important to acknowledge that this 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.

9. This document is divided into sections covering:

- guidance for carrying out a preliminary assessment of test chemical stability;
- general considerations on selection of exposure systems;
- stock and test solution preparation and exposure systems;
- sampling of test solution for analysis; and
- calculating and reporting test results.

Table 1. Properties of "difficult" test chemicals

Property	Nature of difficulty
Poorly or sparingly water-soluble	• Achieving/maintaining required exposure concentrations • Analysing exposure concentration
Toxic at low concentrations	• Achieving/maintaining required exposure concentrations • Analysing exposure concentration
Volatile	• Maintaining exposure concentrations • Achieve water saturation • Analysing exposure concentration
Photo-degradable	• Maintaining exposure concentrations • Toxicity of breakdown products
Hydrolytically unstable	• Maintaining exposure concentrations • Achieve water saturation • Toxicity of breakdown products
Oxidisable	• 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 concentration
Complexing	• Distinguishing complexed and non-complexed fractions in test solution • Depletion of nutrients in test solution
Coloured	• Light attenuation
Hydrophobic	• Maintaining exposure concentrations • Analysing exposure concentration
Ionised	• Distinguishing ionised and non-ionised fractions in test solution • Defining exact exposure concentrations
Multi-component substances	• Chemical characterisation (e.g. identification and quantification of individual chemical components and determination of their properties) • Preparing representative test solutions • Defining exact exposure concentrations

Surfactants	• Assessing phase behaviour in test solution • Maintaining exposure concentrations • Achieve water saturation • Analysing exposure concentration

5. PRELIMINARY ASSESSMENT OF STABILITY OF TEST CHEMICAL

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

- existing data on the physical and chemical properties of the test chemical (for MSs see Section 7.9.2.2);
- existing data on analogous substances;
- data determined from a preliminary stability study carried out under test conditions; and
- existing ecotoxicity studies (acute or chronic) which may provide an indication of behaviour (e.g. dissolution, degradation) of the test chemical under test conditions.

5.1. REVIEW OF EXISTING DATA ON THE TEST CHEMICAL

11. Data relating to the physicochemical properties, fate, transport, and environmental toxicity of a test chemical will be of assistance in selecting a test solution preparation method 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 2. The table includes values for some of the properties which are likely to be indicative of presenting technical difficulties for testing. For other properties, no indicator value could have been set, but such properties should nevertheless be considered with caution due to the experimental difficulties they could trigger.

12. 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 test solution preparation and testing procedures and modifications identified and implemented prior to commencement of testing.

13. Additional considerations for MSs are provided in Section 7.9.

Table 2. Data profile for review of test chemical properties and indicator values of difficulties for test solution preparation and testing

Property	Indicator value [(a)]
Physical state: liquid, solid, gas	-
Chemical characterisation	Require multiple analytical methods to characterise (e.g. MSs) or are otherwise difficult to analyse [(b)]
Molecular weight(s)	- [(b)]
Dissociation constant (pK_a) of an ionisable test chemical	>4 and <10
Maximum logarithmic n-octanol-water distribution constant (logD) of an ionisable test chemical	Maximum logD occurs at pH >4 and <10
Logarithmic n-octanol-water partition constant($logP_{ow}$)	>4
Test chemical saturation concentration in test media under test conditions	<100 mg/L
Water solubility 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	Rapid biodegradation in relation to test duration
Time for primary aerobic biodegradation	Rapid biodegradation in relation to test duration
Soil adsorption coefficient (log K_{oc})	-
Hydrolysis half-life at 25°C within a pH range of 5-9	<24 hours [(c)]
Fish bioconcentration factor (BCF)	>1000 [(d)]
Volatilisation (half-lives) from water: model river and lake	-
Sewage (wastewater) treatment plant removal percentage	-
Photodegradability	Absorbs light at wavelengths 290-600 nm

Dissolution rate and extent for metal compounds	-
Surface tension	<60 mN/m

a. Indicator values are not criteria of difficult test chemicals. They indicate whether these test chemicals are likely to present technical difficulties for testing.
b. Test chemicals which are difficult to characterise analytically are typically high molecular weight, extremely hydrophobic, thermally labile, and/or highly volatile.
c. The unit of time depends on test duration and experimental setup. For example, for longer term chronic tests, a hydrolysis half-life of >24 hours may still result in difficulties maintaining test chemical concentrations.
d. A BCF >1000 is often associated with high hydrophobicity which can lead to difficulties in testing.

5.2. PRELIMINARY STABILITY STUDY

14. If not understood, the stability of the test chemical in the test solution under test conditions should be investigated in a preliminary stability study. A number of physical, chemical, and biological processes can result in significant declines in actual exposure concentrations of a test chemical (e.g. as measured by mass spectral full-scan GC or HPLC chromatogram peak areas), or targeted measures of key or major components for MSs (see Section 7.9) in test solutions over time (see Table 1 and later sections). Where data are absent or insufficient to identify the process responsible for the decline such that corrective actions can be taken, it may be appropriate to carry out a preliminary study for assessing the stability of the test chemical. It should be noted that before testing the stability of the test chemical under test conditions the adequacy of the stock solution preparation method must be investigated prior to initiating chemical testing. Preparation methods which do not result in reproducible stock solutions could result in unacceptable variations of exposure concentrations. As a result of this, uncertainties would exist regarding the actual exposure conditions of a given test chemical. As such, it is paramount that stock solutions be reproducible (i.e. acceptable variation from one preparation to another) and of adequate quality (e.g. water solubility limit is reached when targeted).

15. An example of a design for a preliminary stability study is given in Figure 1. Test solutions of the test chemical are prepared under conditions equivalent (in terms of test medium, pH, test vessels, preparation procedures, etc.) to those to be used in the toxicity test (including aeration if that is envisaged). Stable dispersions and emulsions of the test chemical should only be used in exceptional cases (see Section 7.1.2.3). Samples of the test solution are analysed at the beginning and typically at 24-hour intervals for the duration of the test period. A saturated test solution need not be used for this test but it should contain a detectable concentration of the test chemical or in the case of MSs, major or key components (see Section 7.9). A sufficiently sensitive analytical method is necessary for the analysis of the test chemical in the test solution. The possibility of losses during sampling, sample treatment and analysis must be considered. If samples are to be stored before analysis, storage conditions should be designed to avoid loss of the test chemical during the storage period. Regardless, the stability under storage conditions should be determined. If the test chemical is so sparingly soluble that it cannot be detected using reasonable analytical techniques, it will normally be necessary for the experimenter to use whatever physicochemical data are available in conjunction with expert judgement in order to assess stability. In exceptional circumstances, it may be justified to use specialised techniques (e.g. radiolabelled test chemical) in order to achieve quantitative assessments. It is noteworthy to mention that even if the radioactivity level is maintained, test chemical can still be lost from the test solution (e.g. radioactivity may be associated with degradation

products). The identity of the radiolabelled chemical should be verified as being that of the test chemical at the beginning and during the study.

16. The significance of volatility can be assessed by comparing concentrations in open and closed vessels. Methods to reduce volatile losses are described in Section 7.2.

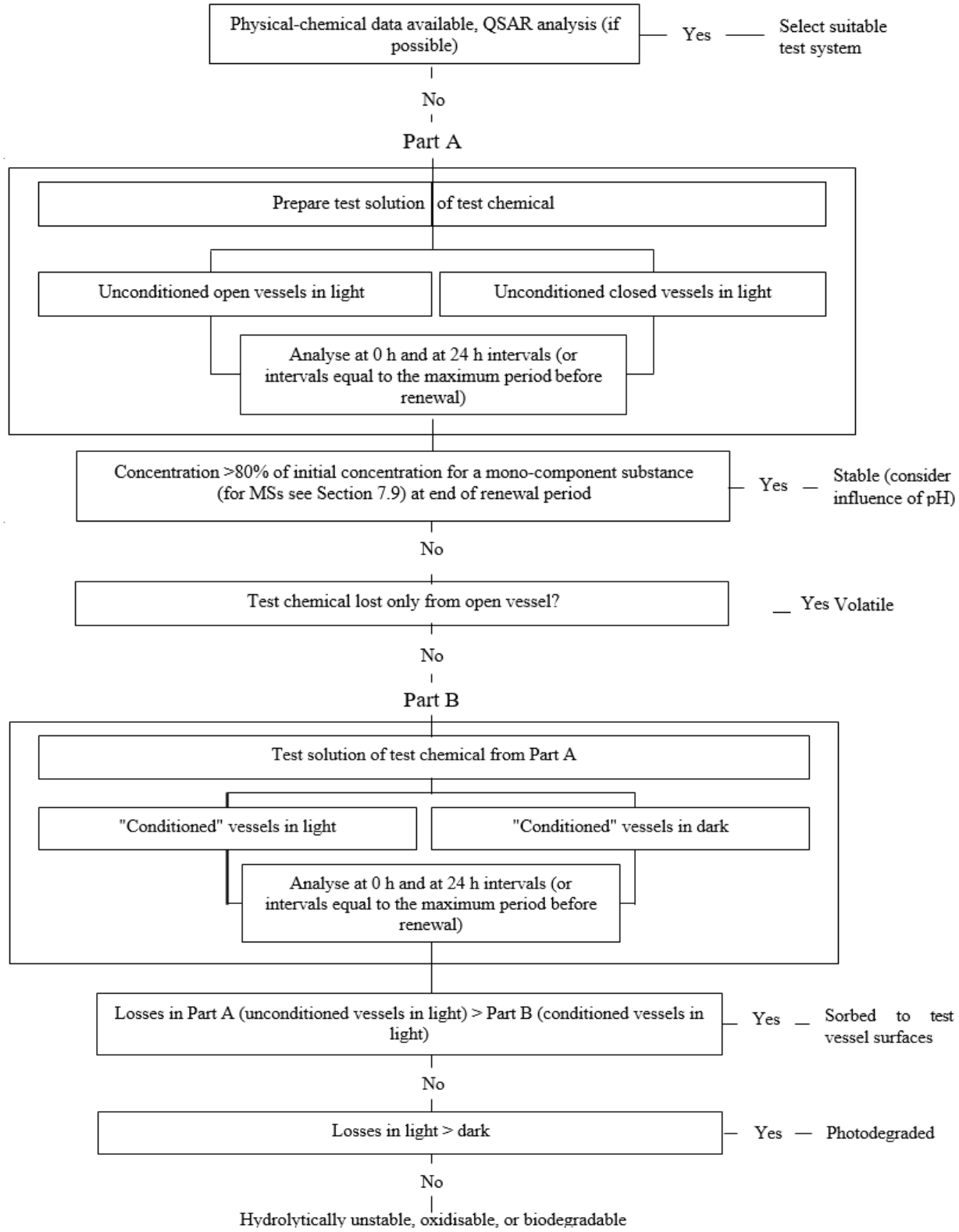
17. The significance of adsorption onto test vessel surfaces can be assessed by a comparative study of concentrations determined in unconditioned closed vessels (see Part A of Figure 1) and in closed vessels conditioned (e.g. using silanising agents) to reduce adsorption (see Part B of Figure 1). Methods to reduce partitioning onto test vessels are described in Section 7.4.

18. If conditioning of test vessels has no appreciable impact on maintaining measured chemical concentrations, then it is most likely that the loss process is a result of photolysis, biodegradation or hydrolysis. However, losses may also be due to oxidation. Photodegradation can be distinguished from a comparison analysis of test solution held under conditions of light and darkness. Greater losses in the test solution exposed to 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 7.3.

19. 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, for example, the US EPA (2016a), Auer et al. (1990), Lynch et al. (1994), Boethling and Nabholz (1997), and Newsome et al. (1996). Preliminary stability studies are not intended to be limited to laboratory studies.

20. 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 chemicals in the test solution. For example, complexation may occur at high concentrations and losses may be greater (proportionally) at low concentrations than at high concentrations. The presence of the test organisms or feed in the vessels may also affect the stability of the test chemical. It may be necessary to carry out the preliminary study with at least two concentrations in order to identify these problems.

Figure 1. Example design of a preliminary experiment for identifying process(es) responsible for loss of test chemical from test solution



6. GENERAL CONSIDERATIONS ON SELECTION OF EXPOSURE SYSTEM

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

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

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

22. It is not possible to give definitive guidance on criteria for selecting an exposure system since requirements may vary between different regulatory authorities. However, a static exposure system is likely to be appropriate if exposure concentrations can be expected to remain within 80-120% of nominal (see Section 7.9 for special considerations for both soluble and poorly soluble MSs) over the whole test period without renewal of the test solution. Likewise, a static-renewal or flow-through exposure system (either intermittent or continuous replacement) will probably be required where concentrations are unlikely to remain within 80-120% of nominal under static conditions. Where uncertainty exists over the suitability of an exposure system it may be useful to consult with the appropriate regulatory authority prior to commencement of the test. In some cases, analytical trials to evaluate stability under the proposed exposure system may be needed.

23. Renewal of test solutions (static-renewal exposure system) after 24 hours exposure may allow exposure concentrations to be maintained and is a relatively simple modification to the test procedure. More frequent static-renewal is possible but care must be exercised not to excessively stress fragile test organisms, e.g. daphnids or early life stages of fish, by excessive handling. An intermittent flow-through exposure system may reduce the stress imposed on test organisms compared with a static-renewal exposure system. Intermittent flow-through exposure 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) and Lammer et al. (2009).

24. A continuous flow-through exposure system should generally be considered when a static-renewal exposure system with renewal following each successive 24 hours exposure period is incapable of maintaining exposure concentrations. Flow-through exposure systems are discussed further in Annex 5.

25. Static-renewal and flow-through exposure systems are likely to require frequent cleaning of the exposure systems to prevent the accumulation of organic debris and the development of excessive microbial populations. This is especially true in the case of chronic testing, where microbial populations may become prevalent over the course of the exposure. Care should be taken to minimise stress on test organisms caused by cleaning.

Cleaning needs are often increased with the use of solvent to deliver the test chemical, as the solvent provides a source of carbon for microbial growth.

7. STOCK AND TEST SOLUTION PREPARATION AND EXPOSURE SYSTEMS FOR DIFFICULT TEST CHEMICALS

26. The objective in preparing stock and test solutions should be to achieve the required and reproducible exposure concentrations of dissolved test chemical at the start of the test. These concentrations should then be maintained, if technically possible and desired, throughout the test using either static, static-renewal, or flow-through exposure systems.

27. The method used for preparing aqueous stock or test solution is dictated by factors such as the physical state of a test chemical, its saturation concentration in the test solution, the desired range of test concentrations relative to the saturation concentration of the test chemical, and the need to generate suitably concentrated stocks for use in flow-through exposure systems. All reasonable efforts should be made to achieve test solutions containing dissolved test chemical at a stable concentration. 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. The goal should be to achieve exposure concentrations up to the aqueous saturation concentration of the test chemical. Visible non-solubilised test chemical in the exposure vessels is generally to be avoided or minimised. Exceptions are possible when suitably justified (e.g. see two exceptions in Section 7.1.2.3). However, this should be accounted for during the chemical analysis of test solutions (i.e. centrifugation or filtration before analysis).

28. The stock and test solution preparation and exposure methods should be evaluated prior to commencing testing to ensure that the required quantities of test solution can be produced and the required concentrations can be achieved and maintained.

29. If significant adaptations to a test guideline are required, the rational for such adaptations needs to be clearly documented in the study records. It is also recommended to consider regulatory guidance and/or to even discuss the proposed methods with the relevant regulatory authority to ensure the study will be accepted. It is important to include appropriate controls to ensure the suitability of the adaptation, including, if applicable, a positive control with a reference chemical. The adaptations and their potential impact on any of the validity criteria of the test guideline should be fully described in the test report along with the technical challenges encountered with the use of standard testing guidelines.

30. It should be noted that OECD Guidance Document 29 (OECD, 2002) and 98 (OECD, 2008) provide considerations regarding transformation/dissolution of metals and metal compounds in aqueous media and may assist to determine the test solution preparation procedure and hazard assessment of metals and metal compounds. In addition, it may be relevant to consider the same methodology for classification of poorly soluble test chemicals that are known to transform gradually (e.g. via hydrolysis) to substances of higher concern (e.g. as a supplement or alternative to tests based on water-accommodated fractions [WAFs]).

31. When working with MSs refer to Section 7.9 below. Further information regarding the use of flow-through exposure systems is provided in Annex 5.

7.1. POORLY/SPARINGLY WATER-SOLUBLE TEST CHEMICALS

7.1.1. Solubility experiment to determine saturation concentration under test conditions

32. Prior to conducting any testing, a solubility experiment should be conducted for all studies with poorly water-soluble test chemicals to determine the maximum dissolved concentration that can be achieved in the specific test solution under test conditions, which is defined as the saturation concentration, and the preparation conditions that are required to achieve it. The results of the solubility experiment will form a basis of, and justification for, the test solution preparation procedures adopted for the toxicity tests and a reference point (i.e. saturation concentration in test solution under test conditions) against which the test results can be evaluated. In view of its importance this experiment should be fully reported.

33. It should be noted that a high level of uncertainty is often associated with solubility and saturation concentration estimates for poorly water-soluble test chemicals. The maximum achievable dissolved concentration of a test chemical in the test solution, i.e. saturation concentration, may not be the same as the water solubility of the test chemical as determined by, for example, OECD TG 105 (OECD, 1995), as the test solution (containing undissolved organic matter, salts, etc.) is much more complex than distilled water. The saturation concentration can be less in test solution compared to distilled water. It is, therefore, often not possible to specify an upper exposure limit with a high degree of confidence without analytical confirmation. Additionally, it is noted that the pH of the distilled water may be different than that of the test solution and that differences in those pHs may significantly affect the solubility, especially of ionised test chemicals with a pK_a between 5 and 9. Furthermore, it can be logistically challenging to prepare test solution as rigorously or over extended time periods when compared to testing the solubility for physicochemical characterisation. Exposure concentrations which do not achieve a "reported" water solubility should, therefore, not necessarily be considered invalid.

34. The design of the solubility experiment in test solution should take into account the mixing/contact time to achieve maximum saturation concentration and possible need to remove non-dissolved test chemical from the test solution. A minimum mixing/contact time of 24 hours might be considered depending on the properties of the test chemical. Saturated solutions may be prepared in a number of ways including, for example, the direct addition or generator system methods. The use of generator systems is recommended for test chemicals with solubilities $<10^{-2}$ g/L, while the direct addition (i.e. flask method) may be used with solubilities above this value, as stated in OECD TG 105 (OECD, 1995). The recommendations made in the following paragraphs are intended to supplement those in OECD TG 105.

35. Physical separation of dissolved and non-dissolved test chemicals can be facilitated by allowing a settling period and then decanting the aqueous test solution or preparing test solutions in an aspirator flask or separating funnel. It should not be assumed that a clear test solution indicates a true solution since crystals, aggregates, micelles, etc., cannot easily be detected by visual observation. Observing the Tyndall effect by shining a laser light through the solution can be helpful in identifying colloidal suspensions (see for example Heller and Vassy, 1946 and Mengual et al., 1999). Further potential separation techniques include:

- Centrifugation - the preferred separation method but there may be practical difficulties in applying the technique to large volumes. As a guide, centrifugation at 100,000 to 400,000 m/s^2 (approximately 10,200 to 40,800g) for 30 minutes may achieve adequate separation; however, other centrifugation regimes may be applicable. The laboratory should verify the efficacy of any separation method, for example by checking for the Tyndall effect. It should be noted that most centrifuge containers are made of various sorts of plastics which may adsorb the test chemical, and that glass containers are more likely to break. Furthermore, centrifugation may also lead to losses of volatile test chemicals. The temperature during centrifugation should be kept in the range of the test solution temperature due to test chemical stability issues.
- 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 solution are required. Filter pore sizes of 0.22 to 0.45 µm may be suitable for achieving adequate separation, but larger pore sizes may be the only option with large test solution volumes. In some cases it may be necessary to repeatedly filter the solution. The filter matrix should be made of inert materials (i.e. chemically and physically non-reactive with test chemicals). If required, filters should be rinsed with high-purity water prior to use to reduce the risk of contamination of test solutions with potentially toxic residues. Adsorption of the test chemical to the filter may be reduced to insignificant levels by preconditioning filters with solutions of the test chemical prepared at the appropriate test concentrations. Filtration under pressure is preferable to vacuum filtration due to potential losses by evaporation.

36. For liquid chemicals, filtration may not be the best way to achieve separation and other techniques should be considered. If the test chemical is a liquid, then the test solution should never be filtered to dryness since this may encourage the formation of an emulsion of undissolved test chemical in the filtrate. For liquids with a density not equal to 1 g/mL, undissolved test chemical can be separated from the aqueous fraction using a liquid/liquid saturator (see Section 7.1.2.2.3). For liquids with a density close to 1 g/mL, separation techniques must be carefully evaluated.

37. A justification for, or validation of, the separation technique should always be provided in the test report. Particular care should be taken when separation techniques are used to prepare solutions of test chemicals that have surfactant properties.

38. The concentration of the test chemical in the test solution should be confirmed analytically after the separation technique wherever possible. Where the dissolved fraction cannot be analytically measured (e.g. when solubility is below a quantifiable level)[1] regulatory guidance should be sought. For example, options may include providing a statement from an analytical chemist in the study report confirming that the analytical methods used were state of the art, and a justification as to why lower detection limits were not feasible (any preliminary analytical efforts should also be described in the report).

[1] For very poorly water soluble or highly hydrophobic test chemicals exposure to the dissolved fraction via water column may not be the most ecologically relevant route and sediment tests may be considered in some regulatory contexts. In some cases, chronic fish tests may be required for such chemicals to evaluate long term hazard to aquatic vertebrates. Since chronic fish tests typically include a requirement for analytical exposure confirmation, a dietary exposure may be an ecologically relevant exposure option. The OECD TG 305 (OECD, 2012a) has been revised to include a dietary exposure option for highly hydrophobic or adsorptive test chemicals. However, specific guidance for conducting chronic fish tests with dietary exposures is beyond the scope of this guidance document and regulatory authorities should be consulted before proceeding.

Direct addition techniques that allow a nominal concentration (or loading rate) to be defined are preferred.

7.1.2. Test solution preparation methods

39. Four methods of test solution preparation have been applied to poorly water-soluble test chemicals. These methods are referred to here by the generic terms: direct addition, generator systems (saturators and passive dosing), dispersions and emulsions, and water-miscible solvents. Care should be taken due to the fact that stability of a test chemical measured in standard stability studies can differ greatly from that observed in the exposure system in the presence or absence of test organisms.

7.1.2.1 Direct addition

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

- low energy (prolonged) stirring;
- vigorous shaking;
- blending;
- homogenisation/high-shear mixing;
- (ultra-)sonication.

41. Preliminary tests should be used to determine the optimum stirring duration to achieve the practical saturation concentration in the test solution (if required). As a pragmatic approach, stirring for a minimum of 24 hours might be considered depending on the properties of the test chemical. Longer durations can be considered but other factors such as test chemical stability and sorption to the vessel and stirrer bars should be considered especially when longer durations are required and small volumes are stirred.[2] Although relatively long stirring periods can achieve the maximum water saturation concentration, it can also result in the formation of degradation products to an extent that these may induce toxic effects in addition to that of the parent test chemical. Letinski et al. (2002) recommends prolonged slow stirring with no vortex formation for chemicals that are liquids at room temperature to avoid emulsion formation. Test chemicals susceptible to photodegradation should be protected from light during prolonged stirring.

42. (Ultra-)sonication is a powerful method that can facilitate dissolution of solids but can be limited by the test solution volume required. This method may not be suitable for treating large volumes of test solution. A period of 30 minutes has been suggested for (ultra-

[2] For example, polytetrafluorethylene (PTFE) and polydimethylsiloxane (PDMS) were used or shown to selectively extract substances such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) or phthalate esters from water (Li et al., 2002; Cai et al., 2003; García-Falcón et al., 2004; Serôdio and Nogueira, 2006; Gupta et al., 2008). For such test chemicals a glass coated stir bar should be considered. If concentrations are confirmed analytically, extraction of organic test chemicals from the aqueous phase is taken into account.

)sonication, but shorter periods may also be appropriate, because (ultra-)sonication may lead to considerable heating, particularly of small volumes, and potential consequences for stability.[3] The treatment may degrade the test chemical itself and/or generate unintended byproducts such as reactive oxidative species.

43. If the pyrolytic stability and volatility of the test chemical allow,[4] heating of the test chemical and test medium may also help to achieve more rapid dissolution, but care must be taken to ensure that this does not result in changes to either the test chemical or the test medium, which could influence the outcome of the test. After cooling down the test solution to test temperature any undissolved particles should be removed according to the procedures indicated above (Section 7.1.1). Attention should also be given to potential decrease of dissolved oxygen concentration. Justification for the heating process should be included in the study report.

44. Concentrated aqueous stock solutions of some poorly water soluble test chemicals containing ionisable groups (e.g. amines) can be produced by increasing or decreasing the pH of the solution. The stock solution can be diluted to target concentrations in the test medium. Depending on the buffering capacity of the test medium, it may be necessary to adjust the pH of the test solution prior to adding the test organisms to bring it within the required limits for the test guideline and species being used considering also the most toxic or bioaccumulative form of the ionised test chemical (as described in Section 7.8). If the final test solution pH differs by more than 1.5 units from the culture water pH, the test organisms should be adapted to the test medium prior to starting the experiment. In contrast to the use of solvents, when a buffered control is used, an additional unbuffered control is not required. Before conducting tests on animals at extreme pH within the allowable ranges, other methods for preparing test solutions should be considered.

45. Direct addition should be used wherever preliminary studies demonstrate that it is feasible. However, direct addition by weighing may be difficult when preparing low target test concentrations, as may be needed for sparingly water soluble or highly toxic test chemicals. In practice, the lowest amount of the test chemical that can be accurately weighed is approximately 1 mg (depending, e.g. on the nature of the test chemical and the balance used). To achieve test concentrations less than 1 mg/L it may be necessary to prepare large volumes of test solution; however, the homogeneity of a large test solution volume should be carefully assessed particularly if insoluble microparticles may be present. In some cases, diluting a more concentrated stock solution may be the only practical option. If the test chemical is soluble in a solvent, a small volume of this solvent stock solution can be added directly to the test vessel to facilitate handling masses less than 1 mg (see Section 7.1.2.4). If the solvent is completely evaporated before test medium is added, then no solvent control is needed. This approach is similar to a solid-liquid saturator (US EPA, 2002), as described in Section 7.1.2.2.2.

46. If testing up to saturation is necessary, test solution could be prepared by direct addition at higher nominal concentrations than the theoretical water solubility limit to achieve the maximum dissolved concentration provided any non-dissolved test chemical is separated before testing (e.g. see Section 7.1.1). Due to differences in the properties of the

[3] If sonication is used with liquid test chemicals, the test solution should be checked for emulsification as this will lead to unrealistically high concentrations. Moreover, temperature should be controlled to maintain integrity of the test chemical, e.g. by using a temperature-controlled water bath or cycles of sonication and cooling.

[4] Stability information could be available in, e.g. safety data sheets or certificates of analysis.

test chemical and impurities being tested it is not possible to specify an approximate factor to use when preparing solutions by direct addition at higher nominal concentrations than the theoretical water solubility limit for all test chemicals. However, when preparing these test solutions the presence of soluble impurities at high concentrations should be avoided because the impurities may be toxic to aquatic organisms and confound the results of the study.

7.1.2.2 Generator systems

47. Generator systems have to comply with a number of criteria. These encompass the requirements of:

- complete dissolution of the test chemical without the formation of a colloid;
- maintenance of stable and predictable concentration over the test duration;
- full and rapid compensation for losses; and
- the absence of any interference with biological activity, e.g. by avoiding direct contact between test organisms and dosing phase (Brown et al., 2001; Kramer et al., 2010; van der Heijden et al., 2015).

7.1.2.2.1 Saturator columns

48. Aqueous test solutions of poorly soluble test chemicals that are mono-component substances can be produced by bringing test media into contact with a chemically inert matrix which has been coated with the test chemical. A matrix with a high surface area to volume ratio (such as glass beads or glass wool) allows a relatively large amount of test chemical to be coated on the surface and provides a large area over which dissolution of the test chemical can take place. Prior to coating, care should be taken to ensure that there are no manufacturing residues present on the column or matrix by solvent rinses or pyrolytic treatment. The actual coating may be achieved as indicated below for the solid-liquid saturator with a stock solution of the test chemical in a volatile solvent (e.g. acetone). Caution must be taken to ensure that the matrix has been coated evenly and the solvent completely evaporated prior to passing test medium through the system. It is crucial to choose a solvent which dries evenly and without generating condensation within the matrix. Once the functional limit of saturation has been established with a generator system, the size of the generator system and amount of test chemical applied to the matrix can be scaled up or down to accommodate different needs for system dosing. High demand exposure units require high flow rates and often multiples of the column units. The use of high flow rates to obtain larger volumes of stock solution often leads to channeling and a lower yield. It is advisable not to use a saturation column longer than its estimated depletion half-life calculated using the loaded mass, saturation concentration and flow rate. Analytical profiling should be performed to identify column efficiency and to determine the active life of the generator system and document concentration yield. Depending on the physicochemical properties of the test chemical and the amount of test chemical loaded onto the generator system, some systems can deliver saturated test solutions for up to six weeks, limiting the need to prepare dosing systems during an exposure.

49. Systems utilising columns packed with a suitable matrix have been described by Veith and Comstock (1975), Gingerich et al. (1979), Phipps et al. (1982), and Kahl et al. (1999). Moreover, the column elution method is the recommended procedure to establish water solubility for test chemicals with solubility <10 mg/L according to OECD TG 105

(OECD, 1995). The aqueous test solution eluted from the column can be used either directly for testing or diluted to produce a concentration series.

50. Often saturation concentration can be achieved with a single pass through the column once it is equilibrated and using larger columns (e.g. >350 mL) or slower flow rates help to facilitate this. However, it may be necessary to re-circulate a test solution repeatedly through a column in order to achieve a saturated solution prior to use in a test. Generally, the test solution should not be recirculated or recycled after being used for exposure. The latter practice may result in complexation of the test chemical with dissolved organic matter and measured total concentrations of the test chemical exceeding the saturation concentration (Billington, 1988) or losses due to sorption, metabolism, or bioaccumulation. One consideration of the generator column method is the challenge of obtaining large volumes of test solution, especially if testing at the functional limit of solubility is required. If generator systems are to be used for flow-through exposure systems, multiple columns in tandem can be used to deliver the required volume.

51. Saturator columns are not generally considered appropriate for MSs. Differences in water solubility of individual components in the MS will result in selective depletion of the more water soluble components from the generator matrix and their relative concentration in the water phase. This may be equally applicable to mono-component substances with impurities which differ in their water solubility. Saturator columns may also not be suitable for test chemicals which are volatile or subject to hydrolysis or liquid test chemicals which can wash off of the matrix. Appropriate chemical analyses of the test solution generated should verify the adequacy of the generator system for the specific test chemical by demonstrating stable concentrations within 80-120% of nominal or mean measured values.

52. Using saturator columns eliminates the concerns of using solvents and only a single control is required; however, large amounts of test chemical may be needed. At least twice the amount, and often a ten-fold amount of test chemical, is required to successfully implement concentration stability over time, which may be a few days or a week. Control test medium should also be passed through a corresponding blank column.

7.1.2.2.2 Solid/liquid saturator systems

53. A variant of direct addition known as a solid-liquid saturator (US EPA, 2002), involves coating the inside surface of a stock solution container with the test chemical. This is similar in principle to a saturator column.

54. Solid-liquid saturator systems are best used with test chemicals consisting of a single substance (not MS) that are solids at test temperature and sufficiently soluble to be detected analytically. Generally, the test chemical is dissolved in a volatile solvent to act as a carrier and the required mass of test chemical in solution added into the container. The solvent is evaporated while rotating the container to coat the walls with the test chemical. A rolling mill with variable speeds can aid in providing an even coat which helps maintain uniform concentration of the stock solution. When the solvent is completely evaporated, the test medium is added to the container and it is mixed until the desired concentration or saturation is achieved. The optimal mixing time must be determined empirically for each test chemical and the mixing process should not physically dislodge the layer of test chemical (e.g. stir bars).

55. Since the solvent is removed prior to any test organism exposure, a separate solvent control is not needed. Nevertheless, it is highly recommended that a corresponding control container be prepared in parallel with clean solvent for the control water in order to

demonstrate the inertness of the test system. As with coated columns, the amount of chemical needed to achieve long-term yield of stock solution from the container coating process is also multiples of that needed to conduct the exposure.

7.1.2.2.3 Liquid/liquid saturator units

56. Liquid/liquid saturator systems as described in Kahl et al. (1999) and Letinski et al. (2002) can be used to generate test solutions, at or near the saturation limit for use in aquatic toxicity tests. This method is applicable to test chemicals with a melting point below ambient temperature and involves slowly stirring excess test chemical and test medium at a constant rate. The units may be prepared as static systems or Kahl et al. (1999) describe how such systems may be adapted to flow-through exposure systems. Successful saturator operation requires identification of the optimal contact time of the test medium in the vessel. The flow of test medium through the unit and its contact time increase or decrease efficiency. Maximum efficiency can be determined by analysing the concentration of the solution at various flow rates. As the flow rate increases, a point of diminishing return will occur rendering the mixing unit inefficient. If this technique is used for MSs, each concentration must be prepared separately as recommended for WAFs and only the static system is recommended with these test chemicals.

7.1.2.2.4 Passive dosing

57. Passive dosing is a technique for establishing and maintaining concentrations of poorly soluble chemicals (i.e. hydrophobic organic chemicals) in aquatic toxicity tests. A biocompatible polymer is first loaded with the test chemical and then included in the test system where it acts as a partitioning donor that controls exposure concentrations throughout the test (Mayer et al., 1999; Smith et al., 2010; Butler et al., 2013). Because this is a relatively new approach used in aquatic toxicity tests, the technique is described in detail in Annex 6. Regulatory authorities should be contacted prior to conducting aquatic toxicity tests using passive dosing to ensure acceptability of the results.

7.1.2.3 Dispersions and emulsions

58. 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 the dissolved test chemical;
- The presence of non-dissolved test chemical presents significant difficulties for the determination of exposure concentrations; and
- Non-dissolved test chemical present in the test solution has the potential to exert physical effects on test organisms which are unrelated to chemical toxicity.

59. 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. In these cases, the rationale for conducting non-standard tests should be clearly documented in the study records. It is also advisable to contact the appropriate regulatory authority to

ensure that the study will meet regulatory requirements. Specific non-standard testing guidance on the testing of test chemicals in the presence of chemical dispersing or emulsifying agents may also be sought from the appropriate regulatory authority.

- Where the test chemical 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 test chemical/litre or the dispersibility limit (i.e. the limit at which phase separation takes place), whichever is lower.[5]

60. Stable dispersions or emulsions can sometimes be produced by the simple expedient of physically mixing the test chemical with the test medium without the use of auxiliary agents such as chemical dispersants or emulsifying auxiliary agents. These auxiliary agents are not advocated because of the potential for physical or chemical interactions influencing the apparent toxicity of the test chemical. Appropriate solvents used in conjunction with physical mixing may also be effective, particularly where the test chemical is a solid.

61. A dispersion or emulsion should be stable before the test organisms are introduced. Any excess of test chemical not uniformly distributed throughout the test solution should be removed[6]. No attempt should be made to maintain test chemical in suspension, by for example stirring, except where this is required by specific test protocols. A static-renewal exposure system should be considered if a stable dispersion or emulsion cannot be maintained for the duration of the test.

62. The potential for non-dissolved test chemical 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 chemically-mediated toxicity. Techniques for physically separating the test organisms from non-dissolved test chemical, whilst maintaining contact with the water column, should be considered where physical effects are likely to be significant (see Section 7.7).

63. In tests with dispersions or emulsions of mono-component substances, exposure concentrations should be expressed in terms of the concentration of the test chemical in solution in order to express the intrinsic toxicity of the test chemical, otherwise the toxicity may be underestimated.[7] Dissolved concentrations can be approximated by measurement following separation of the non-dissolved test chemical from the aqueous phase (see Section 7.1.1).

64. However, where the mono-component substance is self-dispersing in water forming micelles, micro-dispersions, and macro-dispersions, such as certain surfactants, some charged polymers, and many aliphatic amines, exposure concentrations should be expressed in terms of the whole test chemical dispersed in test solution equivalent to the nominal concentration. It is therefore recommended to consult regulatory guidance or even

[5] Where a supersaturated dispersion exerts no effects (no toxic or physical effect), results are regarded as acceptable.

[6] For liquids with a density not equal to 1 g/mL, undissolved material can be separated from the aqueous fraction using a liquid/liquid saturator (see Section 7.1.2.2.3). For liquids with a density close to 1 g/mL, separation techniques must be carefully evaluated.

[7] Given that there are no physical effects due to undissolved test chemical in suspension.

the regulatory authority to decide how exposure concentrations are to be expressed. For MSs, see Section 7.9.

65. Toxic effect concentrations for dispersions and emulsions should be compared with the dispersibility limit (see for example, Harkins et al., 1930, which includes a chapter on surface tension measurements) or the critical micelle concentration (Haftka et al., 2016) for a test chemical in water rather than with its water solubility limit.

7.1.2.4 Solvents

66. Water-miscible solvents provide a vehicle in which some poorly soluble test chemicals can be dissolved to produce a stock solution which is more amenable to adding to, and mixing with, the test medium. In particular, solvents could be helpful for hydrolytically unstable and highly viscous test chemicals. However, because of the potential for interaction with the test chemical resulting in an altered response in the test, the possibility of microbial growth and effects on water quality, e.g. dissolved oxygen levels, solvent use should be restricted to situations where no other acceptable method of test solution preparation is available (Weyman et al., 2012).

67. Currently, when solvents are used two control groups are required: one in the presence of and one in the absence of solvent. For tests involving animals, this has animal welfare implications that should be considered when developing the study design. If solvents are used, their effects on the test results, if any, need to be determined and results for controls in the presence and absence of the solvent should be reported. It should be emphasised that solvents are generally not appropriate for MSs, where the use of the solvent can give preferential dissolution of one or more components and thereby affect the toxicity.

68. The choice of solvent will be determined by the chemical properties of the test chemical and the availability of data to demonstrate that the solvent does not affect the outcome of the study for a given test guideline and species. Solvents which have been found to be effective for aquatic toxicity testing include acetone, ethanol, methanol, tertiary-butyl alcohol, acetonitrile, dimethyl formamide, dimethyl sulfoxide, and triethylene glycol. The physicochemical properties and aquatic toxicity of a range of solvents have been reviewed by Tarr and Hutchinson (1992) and ECETOC (1996). The concentration of the solvent in the test solution 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 0.1 mL/L (Green and Wheeler, 2013). However, some interactions cannot be excluded (Ball, 1996; Hutchinson et al., 2006). For instance, it should be noted that the use of solvents such as acetone, ethanol and methanol can be problematic for static and static-renewal exposure 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 exposure systems. It is unlikely that a solvent concentration of 100 mg/L will significantly alter the maximum dissolved concentration of the test chemical which can be achieved in the test solution but it should be confirmed that tests are not conducted above the saturation concentration (Weyman et al., 2012). Furthermore, reasonable efforts should be made to reduce the solvent concentration to minimise the potential for any effects or interactions (Green and Wheeler, 2013). For example, there is some suggestion that, especially for endocrine screens (for example, OECD TGs 230 [OECD, 2009a] and 231 [OECD, 2009b]) and fish reproduction studies

(for example, OECD TGs 229 [OECD, 2012b] and 240 [OECD, 2015]), a solvent concentration not exceeding 0.02 mL/L should be used (Hutchinson et al., 2006) to account for potential impacts on biomarker endpoints.

69. The recommended approach to test solution preparation is to first prepare a concentrated stock solution of the test chemical in the solvent. Measured quantities of the stock solution are then added gradually to the test medium whilst continuously mixing using an appropriate technique or delivered into the mixing chamber of a suitable flow-through diluter system. The accuracy of the dosing system should be ensured prior to commencing the study. 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.

70. When using auxiliary solvents to aid in the dissolution of the test chemical, preliminary work should be undertaken to assess the dissolved, and hence bioavailable, test chemical concentration as it should not be assumed that all of the test chemical may easily be fully dissolved. Test chemicals 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 exposure system, using a solvent stock solution of the test chemical for dosing the test medium, may not give sufficient time for the test chemical 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. Methods such as filtration and/or centrifugation of samples as previously described must be undertaken to confirm the dissolved test chemical concentration. It should be noted that such solvent systems are not appropriate for metals.

71. OECD guidelines for aquatic toxicity tests for all exposure systems require that the concentration of the solvent must be the same in all treatments (including where technically feasible in flow-through exposure systems). The test design should include a control group of organisms which are exposed to the highest concentration of the solvent used in any of the treatment groups.

72. The use of volatile solvents which can be stripped from the test solution after mixing has also been referred to in ISO 5667-16 (ISO, 2017). The potential for co-stripping of volatile test chemical from, and the retention of toxic solvent residues in, the test solution are likely to restrict the applicability of this approach. However, these latter points should be considered when using volatile solvents to prepare solid-liquid saturators or columns.

7.2. VOLATILE TEST CHEMICALS

73. The vapour pressure (*vp*), water solubility, and the calculated or measured Henry's law constant (*H*) of a test chemical are important parameters determining the potential for a test chemical to be lost from test solutions to the atmosphere by evaporation. These parameters, along with volatilisation from water, and environmental partitioning based fugacity models can be obtained for a test chemical using publically available programs, such as EPI Suite™ (refer to Annex I for more information). See Section 7.9 for the use of subcooled corrected values for solid components of MSs.

74. Vapour pressure is a measure of the equilibrium between the condensed and vapour phases of a test chemical.

75. The Henry's law constant for a test chemical is a measure of the concentrations of chemical between an ideal solution phase and the vapour phase at equilibrium. As such it is a measure of the potential for a test chemical to be lost from solution by evapouration. 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:

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

76. As an approximation, if *H* is greater than 100 Pa.m^3/mol, more than 50% of the test chemical could be lost from the water phase within 3-4 hours (Mackay, 1991). However, other factors in the exposure system may affect the rate of loss, principally test vessel size and shape, headspace volume, depth and temperature of the test solution and rate of aeration. The losses due to volatilisation may become significant for test chemicals 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.

77. Losses of volatile test chemicals from test solution during preparation and exposure can be minimised using relatively straightforward modifications to procedures. If the vapour phase concentration is below the equilibrium concentration, there will be increased tendency for the chemical to leave the aqueous phase and move to the vapour phase. Therefore, anything that will maintain the vapour phase concentration is desirable. As a general rule, test vessels should be sealed during preparation and exposure and the headspace kept to a minimum or eliminated. Likewise, for readily soluble test chemicals, test concentrations should, where possible, be prepared individually by addition of test chemical 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 test chemicals (in water miscible carrier solvents, if required) into sealed vessels. Samples collected for analysis should be placed in zero headspace vials.

78. A tiered approach, along the lines of the following, is suggested for selecting an appropriate exposure system optimal for testing chemicals from the least to most volatile:

a. Open system, no test solution renewal, analytically determined exposure concentrations;

b. Open system, static-renewal of test solution, analytically determined exposure concentrations;
c. Open system, flow-through test solution replacement, analytically determined exposure concentrations;
d. Closed static system, with or without headspace, analytically determined exposure concentrations;
e. Closed static-renewal or continuous flow-through system, with or without headspace, analytically determined exposure concentrations; and
f. Closed static-renewal or continuous flow-through system, no headspace, nominal exposure concentrations.

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

80. The maximum exposure concentration must not exceed the saturation concentration of the test chemical. If it is not the case, the approaches adopted should be consistent with those described in Sections 7.1.2.3 (dispersions and emulsions) and 7.9 (MSs).

81. The following comments relate to specific tests:

- Fish tests: Depletion of dissolved oxygen from test solutions 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, or more frequent test solution renewal (static-renewal or flow-through) in order to maintain oxygen concentrations within guideline values. When using pure oxygen for aeration of the test solution, the pH of the test solutions should be monitored. Dosing systems for flow-through exposure systems have been described by Mount and Brungs (1967), Benville, and Korn (1974). More recently, exposure systems for long-term multi-generation growth and reproduction tests have been described in US EPA OCSPP 890.2200 (US EPA, 2015), OECD TG 240 (OECD, 2015), and references therein.
- *Daphnia* reproduction test: Removal of progeny and renewal of the test solution 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 provided test volumes are not too small and the test solution are changed relatively frequently, e.g. daily. Flow-through exposure systems for *Daphnia* chronic tests have been described in ASTM (2012), Diamantino et al. (1997), Sousa et al. (1995), and US EPA (2016b).
- Algal/aquatic plants test: Algal and aquatic plant (e.g. *Lemna*) tests with very volatile test chemicals are technically very difficult to perform satisfactorily and may as a consequence yield results that are difficult to interpret. Guidance for algal growth inhibition tests has been given in ISO 14442 (ISO, 2006) as well as in OECD TG 201 (OECD, 2011), and for *Lemna* sp in OECD TG 221 (2006). 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 test solution. The same generally holds true for exposure systems with aquatic plants; although *Lemna* require a headspace with a relatively large surface area. Halling-Soerensen et al. (1996) have described a system for testing volatile test chemicals 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 CO_2 is maintained in solution by utilising a sodium bicarbonate ($NaHCO_3$) buffer system. Whatever system is adopted it is advisable to establish

that acceptable control culture growth can be achieved before starting the test. Laboratories that utilise non-conventional exposure systems should conduct a test with a reference substance to confirm that the results are suitable for purpose and/or have historic data available demonstrating adequate control performance in the modified exposure systems. The reference substance results should be reported along with test chemical results.

7.3. TEST CHEMICALS THAT DEGRADE IN THE TEST SYSTEM

82. The preliminary stability assessment study outlined in Section 5.2 should consider the stability of the test chemical in test solution. If the test chemical is likely to be unstable, a decision to test the parent test chemical 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 static-renewal exposure systems with test solution renewal intervals of 24 hours:

- Half-life >3 days: test parent chemical;
- 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.

83. These criteria are based on the assumption that problems associated with maintaining exposure concentrations are often more noticeable in static and static-renewal exposure systems compared with flow-through exposure systems. Loss of 20% of the initial concentration of a test chemical 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.

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

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

86. Testing of degradation products will normally be required where the results of a preliminary range-finding experiment or a (Q)SAR analysis indicates that the degradants 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 test solution. Leaving a stock or test solution of the parent test chemical for a period equal to 6 degradation half-lives of the test chemical will generally be sufficient to ensure that the test solution contains only degradation products. The pH of the test solution after allowing for degradation should be neutralised to that of the control test medium prior to testing.

87. Test results should initially be compared on the basis of the concentrations of the parent test chemical used in preparing the test solutions. In this way the relative toxicity of the parent test chemical and degradation products can be assessed. Identification and quantification of the degradation products may subsequently be necessary to aid in interpreting test results.

88. The choice of exposure system in aquatic tests with degradation products needs to take into account water quality considerations as well as chemical concentration stability. Where toxicity exists when testing the parent chemical, and the degradation products have been clearly identified, it may be preferable to determine their toxicity separately, where technically feasible. 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 test chemical. Samples taken for analysis should be treated and/or stored in an appropriate manner to prevent further degradation prior to and during analysis.

89. A useful bench-mark when designing flow-through exposure systems is that, for a test chemical 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. The impact on test organisms should also be considered because very high system renewal rates may cause disruption or damage to the test organism.

90. It should be noted that when selecting exposure systems and test vessels for unstable test chemicals, the tiered approach suggested for volatile test chemicals (see Section 7.2) is applicable. Selection of the appropriate exposure systems should be dictated by the goal of maintaining test chemical concentrations as close to nominal as possible.

7.3.1. Photolysis

91. 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. 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.

92. Performing algal tests in darkness is not possible due to the importance of light for photosynthesis. In algal tests it may be possible to determine the toxicity of the parent chemical using an approach based on selective removal of wavelengths responsible for photolysis from the illumination source of light 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.

93. The use of flow-through exposure systems should also be considered for photo-degradable test chemicals. 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. Flow-through exposure systems for *Daphnia* chronic tests have been described in ASTM (2012), Diamantino et al. (1997), Sousa et al. (1995) and US EPA (2016b).

94. It should be noted that some chemicals, such as PAHs and other petrochemicals can undergo photolysis to result in more toxic degradants. As such, depending on the test chemical, it may not be appropriate to limit photolysis when the toxicity of degradants needs to be considered. In such cases, it may be necessary to conduct tests with and without limited photolysis to evaluate the toxicity of parent chemical and degradants. As noted above, testing of degradation products will normally be required where the results of a preliminary range-finding experiment or a (Q)SAR analysis indicates that the degradants have significant toxicities or other relevant properties (e.g. low or no degradability).

7.3.2. Hydrolysis

95. Exposure concentrations of the parent test chemical should be maximised by keeping the duration of the test solution preparation stage to a minimum. Direct addition of the test chemical to the test medium combined with methods to achieve rapid dissolution is 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 7.1.2).

96. Flow-through exposure systems are not considered appropriate for chemicals 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, although the number of test chemicals in this category is low. These test chemicals should be added directly to the exposure vessels, and vigorously mixed. All test organisms need to be added as quickly and safely as possible after test chemical addition within the time-scale required to maintain the health of the control organisms (e.g. 10 minutes). If the test chemical is too insoluble to achieve a homogeneous dispersion after vigorous mixing, it should first be diluted in a minimum of 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.

97. Test solutions of hydrolysis products of test chemicals which polymerise should be prepared by adding the test chemical very slowly to a vessel which is part-filled with test media and being stirred rapidly so as to avoid locally high concentrations of polymerisable material. Once the test chemical has been added, the vessel should be topped up with test media to the required volume and stirred continuously for a period sufficient to ensure complete hydrolysis. This procedure should enable a test solution of the hydrolysis products to be produced without the formation of polymers.

98. Temperature and pH can influence the rate of hydrolysis of some test chemicals. Adjustment of these parameters, within the range permitted for the test, may therefore be appropriate in order to optimise exposure concentrations of the parent test chemical. 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.

7.3.3. Oxidation

99. Oxidation is an abiotic transformation process which can result in breakdown of chemical structure and have consequent effects on the toxicity of a test chemical. 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 test chemicals which are subject to oxidation.

100. Oxidation cannot be prevented in oxygenated aqueous test media. Maintenance of dissolved oxygen concentration is therefore a key consideration for fish and invertebrate testing. Selection of a static, static-renewal or flow-through exposure systems 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 test solution, increasing the volume of test solution and/or reducing the test organism loading or increasing the rate/frequency of test solution renewal. Flow-through exposure systems may often be appropriate. Stock solutions of the test chemical should be kept under anoxic conditions (e.g. under nitrogen) until introduced to the test medium.

7.3.4. Biodegradation

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

102. 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 chemical. It is also important that carryover of old test solution is kept to a minimum and that test vessels are thoroughly cleaned and, where possible, sterilised at test solution renewals. Antibiotic use is to be avoided. If antibiotics are used, this should be adequately justified. An antibiotic control will also need to be added to the experimental design.

103. A flow-through exposure system with sufficient test solution 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 test chemical (Tolls et al., 1997). Good hygiene procedures will also help to minimise the development of high bacterial populations on surfaces in the exposure system. Using solvents to prepare test solutions should be minimised, and if possible avoided, as their presence will encourage microbial growth.

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

7.4. ADSORPTION OF TEST CHEMICALS

105. The addition of a test chemical to an exposure system may result in the chemical being adsorbed to vessel surfaces or to suspended particulates, which can decrease the dissolved test chemical concentration. In most cases these adsorptive losses will only be important where relatively low concentrations of a test chemical are being tested (e.g. <1 mg/L). (Adsorption or complexation of test chemical with organic carbon [DOC, TOC] is addressed in Section 7.5.) The most common adsorption mechanisms responsible for losses are as follows:

- adsorption to test vessels (typically made of glass or plastics such as polystyrene) via hydrogen or ionic bonding between the test chemical and hydroxyl groups on the vessel surfaces. Glass surfaces offer negatively charged hydroxyl groups which can bind with cationic test chemicals such as surfactants. Salt bridges, formed by divalent cations, such as Ca^{2+} and Mg^{2+}, may also be responsible for binding anionic test chemicals. The sorption to plastics is especially a problem in the often used 24-well or other well titer plates (Riedl and Altenburger, 2007, and Schreiber *et al.*, 2009). This type of absorptive loss removes the test chemical from the vessel, thus reducing the available exposure concentration;
- adsorption to negatively charged biological material, such as algal cells, by the same mechanisms described above. This type of adsorptive loss does not remove the test chemical from the vessel but it may have the effect of either reducing or increasing the available exposure concentration.

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

- reducing the surface area to test solution volume ratio;
- increasing the frequency or rate of test solution renewal in static-renewal and flow-through exposure systems;
- construction of exposure systems using low adsorptive materials such as polytetrafluoroethylene (PTFE), and avoiding use of highly adsorptive materials in the construction of exposure systems (in particular, rubber and polyethylene should never be used as part of exposure systems for testing organics in any situation);
- testing of metals in plastics (e.g. high-density polyethylene [HDPE] or polypropylene [PP]) is generally preferable to glass that may undergo cation exchange processes (e.g. soft soda lime glass is not recommended, but borosilicate glass may be acceptable). PTFE system components are highly recommended where economically feasible. References should be consulted to determine appropriate materials to use for specific metals (Struempler, 1973; Batley and Gardner, 1977; Subramanian et al., 1978; Massee and Maessen, 1981; Rochman et al., 2014);
- pre-conditioning of test vessels using solutions of the test chemical. The concentration of the test chemical used to condition a vessel should not exceed the test concentration appropriate to the vessel; otherwise the test chemical may desorb during the test and increase the exposure concentration. It should be noted that any flow-through exposure system should always be equilibrated with the test chemical, regardless of whether an adsorptive test chemical is used, and confirmed by the pre-test samples on e.g. two days at least. Pre-conditioning vessels for testing of metals with a high gill binding potential from the Biotic Ligand Model (BLM) is recommended (Niyogi and Wood, 2004);

- maintaining dissolved total organic carbon concentrations (other than that due to the test chemical) in all test solutions at or below 2 mg/L, unless special studies are being conducted;
- feeding of fish a few hours before test solution renewal in static-renewal exposure systems (in this case, test chemical concentrations in test solutions should be determined prior to feeding fish),
- removing excess food from test vessels after feeding has finished; and,
- using static-renewal or flow-through exposure systems instead of a static one.

107. Test chemicals which sorb onto glass are usually very hydrophobic. Adsorption losses of test chemical 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.

108. 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.

109. To investigate sorption on algal biomass, algae may be removed from test solution samples for separate analysis and/or abiotic replicates included in the study design to better characterise exposure levels. Alternatively, it may be possible to use passive sampling to measure the freely dissolved concentration of the chemical in the test solution without separating the algae and test solution phases (Hawthorne et al., 2009; Maruya et al., 2009; Lydy et al., 2014; Jahnke et al, 2012). The regulatory authority should be consulted for acceptance of the passive sampling technique.

110. Adsorption may also be a problem in chronic daphnid studies where test chemical adsorbed to the food algae can lead to apparent reduction in the freely dissolved concentration (when algae are separated prior to analysis), but would still provide a secondary exposure route via ingestion. In such cases it may be desirable to determine how much chemical is in both phases (test solution and algae) to explain observed toxicity to daphnids. The methods described in the preceding paragraph are also applicable in daphnid studies to investigate test chemical adsorbed to food algae.

111. Additional information on the toxicity mitigation for cationic test chemicals by TOC/DOC adsorption and complexation is found in Section 7.5 and Annex 3.

7.5. COMPLEXATION OF (OR BY) TEST CHEMICALS

112. Media specified for culturing and testing of organisms 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. In addition, water hardness can influence the toxicity of metals (Sprague 1995) and ionic organic chemicals (Marchand et al, 2013). As such, hardness should be measured at least at the beginning and end of a given test, renewal interval, or more frequently if changes in hardness are expected. Thus, the composition of culture and test solution may require special consideration to ensure that test results correctly reflect the toxicity of metals, metal compounds, and ionic organic chemicals. Chemical complexation reactions in the test

solution can affect toxicity by, a) various components (DOC and chelators, such as EDTA) reducing the bio-availability of the dissolved test chemical;[8] or b) the test chemical (e.g. EDTA) reducing the availability in the test solution of salts (such as calcium and magnesium) and trace elements which are essential for supporting healthy test organisms (especially algae). As indicated in test guidelines (e.g. OECD TG 211 [OECD, 2012c] and OECD TG 201 [OECD, 2011]), the use of culture media that does not contain known chelating agents (i.e. EDTA) is recommended when testing materials that contain metals.

113. 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 test chemicals and for extrapolating to a predicted no effect concentration for risk assessment unless additional tests are conducted to attempt to determine the nature and extent of the effect. The extent to which complexation affects toxicity therefore should be determined where possible.

7.5.1. Complexing of test chemicals

114. The extent to which a test chemical is complexed will depend upon various factors in the test solution (e.g. DOC, alkalinity, hardness, pH, other inorganic ions), whether present naturally or deliberately added/manipulated. The toxicity of metal cations (e.g. copper) are perhaps best known in this regard. However, pH can greatly affect the toxicity of weak acids and bases (e.g. ammonia, pentachlorophenol) by the reactions of these chemicals with hydrogen and hydroxyl ions, DOC can affect the bioavailability of hydrophobic and cationic organic test chemicals, and hardness can affect the toxicity of anionic organic test chemicals.

115. Thus, for some regulatory applications it may be necessary to assess the extent to which the toxicity of the test chemical varies with such complexing agents based on controlled experiments which manipulate these factors. For DOC effects on cationic test chemical toxicity, such experiments are discussed in Annex 3.

116. Even if toxicity can be related to a particular chemical species of the test chemical (e.g. uncomplexed or "free" chemical), analytical methods capable of distinguishing this species may not always be available or feasible. As such, addressing the effects of complexation will depend on empirical or mechanistic modelling efforts that interpret the observed dependence of toxicity on complexing factors. For example, for weak acids or bases, the basis for such models will be the pH-dependence and the relative toxicities of the ionised and un-ionised forms, although other factors might also enter into such models. For DOC complexation of organic test chemicals, various models for such complexation can be applied.

117. Complexation of metals to organic and inorganic ligands in test solution and natural environments (including consideration of pH, hardness, DOC, and inorganic test chemicals) can be estimated using metal speciation models such as MINTEQ (Brown and Allison, 1987), Visual MINTEQ (Gustaffson, 2017), WHAM (Tipping, 1994) and CHESS (Santore and Driscoll, 1995). 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; this model also addresses how competing cations affect the binding (complexation) of the toxic metal to physiological receptors (Niyogi and Wood, 2004). The

[8] For more information on bioavailability concepts for metals and inorganics see OECD GD 259 (OECD, 2016).

models used for the characterisation of metal complexation in the test solution should always be clearly reported, allowing for their extrapolation back to natural environmental conditions, and assessments should be based on measured concentrations relative to the models (e.g. dissolved metal concentrations).

7.5.2. Complexation by test chemicals (algal tests)

118. The toxic effects of metal complexing test chemicals in algal growth inhibition tests are mainly caused by reduction of the free (bioavailable) concentration of physiologically essential ions. Inhibition of algal growth by such complexation is a secondary effect, which cannot be attributed to the inherent toxicity of the test chemical.

119. Guidance on toxicity mitigation testing with algae for chemicals which form complexes with polyvalent metals is given in Annex 4.

120. Reduction of the free concentration of physiologically essential ions by complexation by test chemicals can be determined by quantifying free concentrations of essential ions over the course of the exposure in treatments and controls. Analysis methods for quantifying exposure concentrations, which are capable of distinguishing between the complexed and non-complexed fractions of a test chemical (or complexed or non-complexed fractions of limiting nutrients), 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 whole measured test chemical or nominal concentrations.

7.6. COLOURED TEST CHEMICALS

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

7.6.1. Algal tests

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

123. The need for clear guidance on how to determine the inherent toxicity of coloured test chemicals to algae was discussed (EC, 1996; Justesen and Nyholm, 1998) and approaches were addressed by the ECB (2006) superseded by ECHA (2017).

124. The following adjustments to the standard algae growth inhibition test OECD TG 201 (OECD, 2011) should be applied for coloured test chemicals:

a. The irradiation (light intensity) should be above 120 $\mu E/m^2 sec$, which is the maximum level recommended in OECD TG 201.

b. The light path should be shortened by reduction of the volume of the test solutions from e.g. 100 to 5 - 25 mL or even 1 mL.

c. Sufficient agitation (for example by moderate shaking) should be performed in order to obtain a high frequency of exposure of the algae to high irradiation at the surface of the test solution. However, agitation is not advised for algal species that tend to form clumps upon shaking.

125. For highly light absorbing test chemicals, a modified standard *Lemna*-test (OECD TG 221; OECD, 2006) may be recommended instead of an algal study by some regulatory authorities (see *Plants tests* below).

7.6.2. Plant tests

126. Testing with the floating plant, *Lemna* sp. (OECD TG 221; OECD, 2006), may provide a further alternative for coloured test chemicals since this species is unaffected by the optical properties of the test solution. It may depend on regulatory requirements whether the test can be considered in place of the algal growth inhibition test for coloured test chemicals. For coloured test chemicals, the following modification to OECD TG 221 should be applied: the test should be performed on a black, non-reflecting surface.

7.6.3. Invertebrate tests

127. Observation of small invertebrate test organisms, such as *Daphnia* sp., can be difficult in highly coloured test solution. 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.

7.6.4. Fish tests

128. Observations of fish can also be difficult in highly coloured test solution. Observations may be made easier by transferring the test organisms to fresh test medium containing no test chemical for a short period whilst the observations are made. However, if fish are transferred, it may become impossible to observe some of the subtle sub-chronic observations that would otherwise be observed (i.e. if not transferred). Furthermore, it is recognised that the frequent manipulation of fish can be detrimental and stressful to the organism and may well induce observations and effects which are not related to the test chemical.

7.7. HYDROPHOBIC TEST CHEMICALS

129. Partitioning of test chemicals 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 test chemicals with a high octanol/water partition constant ($\log P_{ow} > 4$) or bioconcentration factor (BCF >1000). High octanol/water partition constants are generally associated with low water solubility and hence losses due to partitioning are likely to be more significant for hydrophobic test chemicals.

130. Methods of preparing test solutions of poorly water-soluble test chemicals have been discussed in Section 7.1. Strategies for maintaining exposure concentrations of hydrophobic test chemicals in test solutions include:

- reducing the ratio of test organism biomass to test solution volume (i.e. the use of a ratio of <1 g/L in fish tests);
- using a static-renewal or flow-through exposure system;
- increasing the frequency or rate of test solution renewal in static-renewal and flow-through exposure systems;
- feeding (e.g. of fish) a few hours before test solution renewal in static-renewal exposure systems (in this case, test chemical concentrations in test solutions should be determined prior to feeding fish);
- removing excess food and detritus promptly after feeding, e.g. 30 min post-feeding;
- maintaining dissolved total organic carbon concentrations (other than that due to the test chemical) in acute tests at or below 2 mg/L;
- extending the exposure period of the test to achieve equilibrium;
- conditioning the exposure system with the test chemical concentration to limit adsorption losses during the test.

131. Many of the above mentioned approaches are not applicable to algal tests.

132. Analyses of exposure concentrations in algal tests with hydrophobic test chemicals may need to take account of the potential for partitioning of the test chemical 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 solution. Separation techniques include centrifugation and filtration; both of these techniques are subject to the comments made in Section 7.1.1 (see also OECD TG 201 [OECD, 2011]). Another approach to measure the exposure concentrations in algal tests is the use of passive sampling. With passive sampling, an organic polymer is equilibrated with the test solution and subsequently analysed to determine the freely dissolved concentration of the test chemical in test solution (Hawthorne et al., 2009; Maruya et al., 2009; Lydy et al., 2014; Jahnke et al., 2012). The regulatory agency should be consulted for acceptance of passive sampling approaches.

133. If a film of hydrophobic test chemical is present on the surface of the test solution, the concentration of the test chemical is above its saturation limit. If this is the case, new test solution with lower concentrations of hydrophobic test chemical needs to be prepared. When testing at a lower test chemical concentration is not possible, alternative tests should be considered and discussed with regulatory authorities. A film of hydrophobic test chemical floating on the surface of test solution 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). Microscopic inspection of immobile organisms should be done to check for test chemical entrapped on their surface.

134. It is advisable to consult Section 7.4, since there is much overlap between hydrophobic and adsorbing test chemicals.

135. It should be noted that for highly hydrophobic test chemicals, aqueous concentrations may not be quantifiable and an aqueous exposure may not be the most environmentally relevant route to characterise toxicity. For such test chemicals, exposure via the sediment or diet are more relevant to the environmental situation (OECD, 2012d;

Pataya 2006) and some regulatory authorities suggest these alternate exposures routes (ECHA, 2017). Before proceeding with a dietary exposure study, the regulatory authority should be consulted to be sure the endpoints generated are suitable for regulatory objectives. Guidance on dietary exposures is beyond the scope of this document, but some guidance is available (OECD, 2012a; US EPA, 2002).

7.8. IONISED TEST CHEMICALS

136. 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 distribution constant of the test chemical, and thus also its bioavailability and measurable toxicity. It is, therefore, essential that the relevant dissociation constants (pK_a values) are known prior to commencement of testing.

137. 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 test chemical, should be considered where the pK_a for the test chemical falls within the pH range of pH 4-10. In general, the logD curve can also be considered for significant changes in the bioaccumulation potential and toxicity within the normal test pH range. The definitive test should be conducted at a pH consistent with the most toxic form (usually the non-dissociated form, but could also be the charged form for cationics) or most bioaccumulative form (e.g. non-dissociated form) of the test chemical whilst remaining within the range required to maintain the health of the control organisms. This may require testing at the pH extremes of the allowable range for test organisms. Extremes of pH can by themselves adversely affect test organisms. If tests are undertaken at modified pH levels, the test organisms should be gradually adapted to the new conditions. The test organisms should be able to achieve adequate control performance standards. A positive control test with a known reference chemical may be helpful to determine if test organisms respond as expected to toxicant stress.

138. Where the test chemical itself causes a change to the pH of the test solution, the pH should be adjusted to lie within the specified range for the test using acid, alkali or other suitable buffer prior to addition of test organisms. 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 chemical. Buffers known to complex with the test chemical should be avoided in order to avoid disturbance of exposure conditions. 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 solution or in the test solution itself, as judged appropriate. In either case the procedure should be applied to all treatments, with the goal to bring all pH values to within an acceptable range of the controls (e.g. ±0.2 pH units).[9] If the final test solution pH differs by more than 1.5 units from the culture water pH, the test organisms must be adapted to the test medium prior to starting the experiment.

[9] OCSPP 850.5400 (US EPA, 2012) and Rendal et al. (2012) may be useful in determining the acceptable pH range for algae tests.

139. Growth of algal test cultures in test solution can cause increase of pH due to consumption of HCO_3 ions. Maintenance of stable pH when testing an ionised test chemical is therefore important to ensure that the balance between dissociated and non-dissociated forms of the test chemical is maintained. Strategies for maintaining the concentration of HCO_3 ions and therefore reducing pH shifts have been discussed in Section 7.2.

140. For surface-active test chemicals, care should be taken that exposure concentrations are below the critical micelle concentration (CMC) determined for the test chemical. This will ensure that test organisms are exposed to the freely dissolved chemical species and not the micelle. Formation of micelles can affect the uptake behavior of the test chemical in test organisms and micelles are less likely to occur in natural waters. See also test solution preparation of dispersions and emulsions in Section 7.1.2.3.

7.9. MULTI-COMPONENT SUBSTANCES (MSs)

141. The recommendations outlined in this section represent best practices at the time this guidance was prepared. Methods and approaches for the assessment of multi-component substances (MSs) including UVCBs are evolving and, as such, best practices may change as well. New approaches should be considered if they provide reliable data and in discussion with the regulatory authority to ensure that they meet the regulatory requirements.

7.9.1. Mixtures/Preparations

142. Testing of specific mixtures or preparations (e.g. pesticide formulations) may be determined by regulatory requirements but may generally be advocated in the following instances:

- Where the toxicity of the mixture or preparation cannot reliably be calculated from the toxicity of its components (Note: If the reason that mixture toxicity cannot be calculated from its components is a lack of data, then consideration should first be given to determining the toxicity for any of the components of the mixture or preparation for which data are lacking, so that the toxicity of the mixture or preparation can then be calculated [GHS, 2015]);
- Where data confirming the calculated toxicity are specifically requested according to a regulatory requirement.

143. Where testing of the mixture or preparation as a whole is required, the approaches identified below in Section 7.9.2 should be considered.

7.9.2. Unknown/Variable composition, complex reaction products and biological materials (UVCBs)

7.9.2.1 Substance identity and composition

144. Most multi-component substances, and in particular UVCBs, are a complex mix of individual components with different physicochemical properties (e.g. water solubility, octanol-water partition constants, melting point, etc) and thus the UVCB substance might

exhibit a range of measured values for these parameters. In some cases, they can be characterised as a homologous series of components with a certain range of carbon chain length/number or degree of substitution (e.g. petroleum), while in other cases, they can be characterised as a heterogeneous mix of different sub-classes of components (e.g. biologicals). Different approaches to test solution preparation and/or testing are required for UVCBs depending upon whether they are fully or partially soluble in the test solution across a proposed range of test concentrations. The approach for dealing with a fully water soluble UVCB is outlined in Section 7.9.2.3.

145. The first step is to determine the composition of the UVCB in terms of its components to verify the identity of the UVCB subject to testing and ensure a suitable analytical method exists to track components of the UVCB throughout the testing. Normally, the regulatory authority or study sponsor will provide this information. The analytical information and spectral data reported should be sufficient to allow for unequivocal identification of the whole UVCB substance or fraction(s) or components which will be subject to testing. A combination of Gas or High Performance Liquid Chromatography (GC or HPLC) with Mass Spectroscopy is usually sufficient. It should be noted that where the UVCB is a salt, it is necessary to employ a technique to identify and quantify the ionogenic components. If only key component(s) (i.e. toxicologically significant) or fraction(s) of the UVCB are determined by the regulatory authority to be the subject of testing, then these may be the subject of subsequent chemical analysis.

7.9.2.2 Physicochemical properties

146. Physicochemical data on the UVCB, fraction(s) or component(s) may help predict behaviour (e.g. solubility, volatility, etc) of the UVCB and its components during the test so that optimal conditions may be used. Normally, the regulatory authority or study sponsor will provide this information. These data may be available for the whole UVCB, fraction(s) of the UVCB (e.g. as a range of values) or for individual component(s) of the UVCB. Caution should be used in interpreting physicochemical values measured on a whole UVCB or fraction as most test methods may not be suitable to accurately determine this information. Where data gaps exist, (Q)SAR modelling and/or existing empirical information of physicochemical data for major (i.e. predominant components) or key (i.e. toxicologically significant) components or fractions (e.g. a range based on members on either end of the fraction based on molecular weight) could be used. Components within a UVCB which are solids at room temperature (i.e. melting point is greater than 25°C) may show an increase in both vapour pressure and water solubility within a UVCB due to changes in intermolecular forces in going from a pure substance to a UVCB. Subcooled correction of both water solubility and vapour pressures should be used for solid components within a UVCB (Liu et al., 2013; Bidleman, 1988).

7.9.2.3 Ecotoxicity testing - general information

147. Test methods consistent with those described for water-soluble test chemicals (e.g. direct addition - see Section 7.1.2.1) are appropriate for UVCBs comprised of components which dissolve fully within the proposed range of test concentrations. UVCB components may be susceptible to variable losses in test solution by volatilisation, degradation, adsorption to vessel surfaces, or other mechanisms discussed in Sections 7.2 to 7.8, and thus consult with the regulatory authority if departures from recommendations in 7.9 are

deemed necessary to prevent such losses. Chemical specific analysis (e.g. usually via GC- or HPLC-MS) of the test solution are usually required to demonstrate attainment of equilibrium and stability of the UVCB during the test. If available, the concentration (e.g. mg/L) of components (key or major) in the test solution with known toxicity values will be useful to verify if they are responsible for the observed toxicity; however, demonstration that concentrations were consistently maintained within 80-120% of the initial or mean measured values over the exposure duration should be based on a comparison of the mass spectral full-scan GC or HPLC chromatogram peak areas (or in a more targeted fashion for major and/or key components of the UVCB). If this cannot be done, then another suitable method should be selected, and discussed with the regulatory authority, to demonstrate that this requirement has been met. Specific analytical methods may not be suitable for all UVCBs and sum parameter methods (e.g. total organic carbon) may be used to demonstrate that consistent exposures were achieved. However, such methods will not demonstrate the stability of individual UVCB components during the test and are limited by relatively poor sensitivity (approximately 1 mg/L).

7.9.2.4 Preparing water-accommodated fractions (WAFs)

148. In a case where a UVCB is only partially soluble in water, a water-accommodated fraction (WAF[10]) can be prepared. The term WAF is applied to aqueous test solution containing only the fraction of a UVCB that is dissolved and/or present as a stable dispersion or emulsion. The process of generating a WAF is conceptually identical to the direct addition method of generating saturated solutions with poorly water soluble test chemicals which is described in detail in chapters 7.1.1 and 7.1.2.1. However, a WAF is specific to an MS since it can contain multiple dissolved components whose proportions depend on individual water solubility and the mass-to-volume ratio of the preparation. Consequently, WAFs must be prepared separately for each dose level (loading rate). Using the data derived from the testing of WAFs for classifying aquatic toxicity is referred to in Guidance Document 27 on *Use of the Harmonized System for the Classification of Chemicals which are Hazardous for the Aquatic Environment* (OECD, 2001). The following text provides guidance on the preparation, testing, and data reporting for WAF test solutions with UVCBs. While not an exhaustive analysis of the stipulations and limitations of this process, a discussion of typical recommendations about test solution preparation is provided.

149. WAFs are prepared individually and not by serial dilution of a single stock WAF. In cases where the UVCB is highly toxic, e.g. the NOELR is less than 1.0 mg/L, it may not be possible to prepare each WAF individually due to the limitations of weighing small amounts of UVCB and/or the large test solution volumes required. In such cases, serial dilution of a single stock WAF may be the only practical approach; however, the reasons for this departure from standard practice should be fully described in the test report.

150. Defined amounts of UVCBs 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

[10] The term "WAF" is used throughout the text, but the guidance described herein is generally applicable to tests with either WAF or water soluble fraction (WSF). A WSF is the result of a WAF that is subjected to a separation step (e.g. centrifuged or filtered through suitable filters) to remove any suspended undissolved emulsified components.

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 to determine that maximum saturation has been achieved. Techniques such as turbidimetry, total organic carbon (TOC) or Infra-red (IR) or ultra-violet or visible (UV/VIS) spectroscopy analysis may serve as preliminary indicators of the progress of mixing and phase separation in range-finding experiments. Chemical specific analysis (e.g. usually via GC- or HPLC-MS) of the test solution is usually required to demonstrate attainment of equilibrium and stability of the UVCB during the test, which can be done based on a temporal comparison of peak areas as described previously. The concentration (mg/L) of components (key or major) in the test solution with known toxicity values would also be useful to verify if they are responsible for the observed toxicity. Given the fact that many liquid UVCBs may form emulsions in solution, an attempt should be made to limit emulsification during test solution preparation by using a slow-stir method (see Section 7.1.2.2.3). If an emulsion cannot be avoided, an attempt should be made to differentiate the components which are present as part of the solution (truly dissolved) versus those that are emulsified. The use of passive sampling techniques (see Section 7.7, Cui et al., 2013; Letinski et al., 2014) for measuring the freely dissolved concentration of components may aid with this, however, the regulatory agency should be consulted for acceptance of passive sampling approaches. Procedures for preparing WAFs have been described by Girling (1989), Tadokoro et al. (1991) and Singer et al. (2000).

151. The dissolved concentration of UVCB should be equilibrated and maximised by mixing while also controlling the mixing rate to prevent the formation of emulsion or suspension of micro-droplets in the aqueous phase. Typically, a slow-stir method where the test solution is stirred at a speed such that a small "dimple" is formed at the test solution surface is sufficient to allow for equilibration within 48 hours; however, other stirring rates may be appropriate for a specific type of UVCB. 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 chemical in the WAF. The Tyndall effect may be used to evaluate the amount and stability of the emulsion over time. If the components of the UVCB degrade quickly, the stirring period will have to be limited to ensure no unacceptable losses occur, and monitoring the UVCB using chemical specific methods or techniques capable of detecting changes in total mass of test chemical (e.g. TOC) at the start and during the test is required (see Section 7.3 on how to address any single component of a UVCB that degrades in the exposure system). Lastly, it is important to note that the presence of water-miscible solvents can modify the composition of a WAF and as a consequence they should not be used during preparation or should be completely evaporated prior to adding test medium.

152. Generally, any non-dissolved test chemical component which has sedimented in the test vessels or formed a surface film should be removed from the test solution using, for example, a separating funnel or by mid-depth siphoning of the aqueous phase. Alternately, this could be done by running-off of the lower aqueous-phase (excluding the aforementioned non-dissolved test chemical) using a mixing vessel with tap located on the bottom (e.g. aspirator flask). Alternatively, any fraction of the test chemical forming a surface film could be decanted to prevent surface trapping. Any remaining non-dissolved test chemical component in the WAF has the possibility to cause physical effects on the test organism unrelated to inherent chemical toxicity. If this is a problem, testing water soluble fractions should be considered. Where there is a need to retain an excess of the UVCB in the test vessels, steps should be taken to prevent trapping or fouling of test organisms (see Section 7.7). The latter approach may be desirable when testing highly

insoluble UVCBs. However, when it is suspected that components of the UVCB may be susceptible to unacceptable losses in test solution as mentioned previously (e.g. via mechanisms discussed in Sections 7.2 to 7.8), consult with the regulatory authority if significant departures from recommendations in Section 7.9 are deemed necessary to prevent unacceptable losses. Also, it is important to provide evidence that all reasonable efforts have been taken to minimise losses and ensure that the dissolved concentration of the UVCB has been equilibrated and maximised with minimal observed emulsion for the duration of the test. This could include, for example, a comparison between mass spectral full-scan GC or HPLC chromatogram peak areas or targeted measures of key or major components within the dissolved and emulsified fractions, when possible, for the different range-finding mixing regimes used and throughout the duration of the definitive test. Due to these special requirements for poorly soluble UVCBs, discussion with the regulatory authority is encouraged to ensure that the study will meet regulatory requirements.

7.9.2.5 Water-accommodated fractions (WAFs) reporting of results

153. Only a fraction of the total mass of the UVCB or other test chemical added to the test solution may be present in the WAF. The term "loading rate" in place of "nominal concentration" has therefore been advocated for expressing exposures of UVCBs 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 UVCB to test medium used in the preparation of a WAF. Results should be calculated from the loading rates of the entire UVCB as either a median lethal loading (LL_{50}) or median effective loading (EL_{50}) value. Similarly, the No Observable Effect Loading Rate or NOELR can be calculated. 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. The measured concentrations of dissolved and/or emulsified key or major components would greatly improve interpretation and also assist in evaluating acceptable loading values for a UVCB, thus, underscoring the importance of chemical-specific analytical determinations. For example, if the concentration of measured components in a WAF increases monotonically with loading rate and effects, this provides evidence that those components may contribute to the observed effects, although non-measured components may also contribute.

154. The method used to prepare the WAF should be fully described in the test report (including visual observations) and evidence of its compositional stability over time should be provided.

155. Newer techniques (e.g. passive dosing - see Section 7.1.2.2.4 and Annex 6) have shown promise in dealing with some of the shortcomings of WAFs for poorly soluble UVCBs. These techniques enhance the stability of the solution, provide more precise control of the concentration of any particular component, ensure that no component is present in excess of the system solubility limits, and prevent the formation of emulsions. The regulatory agency should be consulted for the acceptance of passive dosing.

7.10. ALLOYS

156. In general, alloys can be considered separately from other preparations due to their unique physicochemical properties that differentiate them from simple mixtures of

component 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 test chemicals are not appropriate. However, care should be taken due to the fact that some alloys such as powered brass show toxicity to aquatic organisms.

157. OECD Guidance Document 29 (OECD, 2002) and 98 (OECD, 2008) provide considerations regarding transformation/dissolution of metals and metal compounds in aqueous media and may assist to determine the test solution preparation procedure of metals and metal compounds.

7.11. TESTING AT SATURATION CONCENTRATION OR LOW TEST CHEMICAL CONCENTRATION

158. Testing at the saturation concentration or at low test chemical concentration present unique challenges. This section provides recommendations when testing under these conditions.

7.11.1. Testing at test chemical saturation concentration

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

- the analytical method validation report demonstrating that the analytical method is appropriate;
- the report of an experiment demonstrating that the test solution preparation method is sufficient to maximise the concentration of the test chemical in solution (see Sections 7.1 and 7.9);
- a description of the method used to prepare the test solution;
- a statement of the water solubility; and
- an assessment of saturation concentration.

160. 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 having 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 test chemicals are predicted to have no acute toxic effects at saturation, it is recommended to consult the relevant regulatory authorities. Some regulatory authorities may prefer to omit acute toxicity tests and proceed straight to chronic toxicity testing. See Section 9 for guidance in case exposure concentrations cannot be measured.

7.11.2. Testing at low concentrations

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

appropriate (see Section 7.1.2.4). Effective mixing is essential to ensure that the test chemical is uniformly dissolved throughout the stock solution and test solution. See Section 7.9 for recommendations for dealing with MSs.

162. Exposure concentrations should be confirmed and their stability demonstrated by analysis unless the dissolved concentration is less than the limit of quantification of the most sensitive analytical method. The use of a radiolabelled test chemical may enable very low concentrations to be analysed but this may lead to significant additional costs associated with its synthesis, handling, and analysis.

7.11.2.1 Absence of a suitable analytical method for a mono-component substance

163. In the absence of a suitable analytical method for quantifying exposure (e.g. nominal concentrations are below limits of quantification of most sensitive analytical method), a static-renewal or a flow-through exposure system with as many tank renewals as possible may be necessary to ensure that exposure concentrations are in line with target values. If the exposure cannot be quantified for the relevant concentrations, and the potential for loss processes (e.g. volatilisation, hydrolysis) have been ruled out, the effect concentration can be expressed based on the nominal concentration; however, regulatory authorities should be consulted on the preferred approach. Confirming nominal test chemical concentrations in a stock solution, if used, may provide useful supporting data. See Section 9 for further guidance.

7.11.2.2 Test chemical concentration declines >20% for a mono-component substance

164. See Section 9 for guidance on calculating the concentration that will be used as the basis for assessing effects.

7.11.2.3 Mono-component substance concentrations are below method sensitivity at the end of exposure

165. Efforts should be made to ensure that test chemical concentrations are maintained between 80-120% of nominal. See also additional guidance for test chemicals that are volatile, degrade, or adsorb in Sections 7.2, and 7.4. In addition, see Section 8.1 for guidance on sampling schedules. However, where a measured concentration at the end of the exposure period is absent or where it indicates that the test chemical is not detected, the validity of the test to meet regulatory requirements should be reconfirmed with the regulatory authority. In order to calculate a mean exposure concentration when the test chemical is detected but not quantified in a sample, one possible method is to use a value of half of the limit of quantification. Since there may be various methods for determining the exposure concentration, particularly when concentrations are below the limit of quantification, the method selected should be made explicit in the reporting of test results. It is also advisable to seek guidance from the regulatory authority to ensure that the method meets regulatory requirements.

8. SAMPLING OF TEST SOLUTION FOR ANALYSIS OF EXPOSURE CONCENTRATIONS

166. A detailed consideration of analysis techniques for use in aquatic toxicity tests is outside the scope of this document. Sections 8.1 and 8.2 below are therefore limited to providing guidance on the design of sampling schedules and methods of sampling (see US EPA, 2016a).

8.1. SAMPLING SCHEDULES FOR ANALYSIS OF TEST SOLUTION

167. Selection of the appropriate sampling frequency to confirm exposure concentrations throughout the duration of the test should be dictated by the exposure technique used and the stability and properties of the test chemical. Schedules for collecting samples of test solution for analysis are often included in testing guidelines. The following recommendations are designed to supplement the information given in the test guidelines and provide a lead where guidance is lacking. Note that the sampling schedules outlined in this section may differ from the analytical (minimum) requirements recommended by the respective OECD test guidelines for toxicity testing in order to address the difficult properties of the test chemicals that are the focus in this guidance document.

168. Sampling schedules for static, static-renewal, and flow-through exposure tests are outlined below. These schedules only apply to tests carried out within the solubility range of test chemicals that are mono-component substances. It should be noted that for difficult test chemicals 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 should be noted that in consultation with the regulatory authority it may not be necessary to analyse stored samples below the NOEC of the most sensitive test parameter. Where loses due to e.g. volatility are anticipated, it is recommended that samples for analysis be taken at the beginning of the test, and 24-hour intervals throughout the test in order to obtain the mean measured concentrations.

8.1.1. Static exposure systems

169. A static exposure system is appropriate where exposure concentrations are expected to remain within 80-120% of nominal over the exposure period (see Section 7.9 for special considerations for both soluble and poorly soluble MSs). Analysis of the highest and lowest test concentration and a concentration around the expected test endpoint (e.g. EC_{50}, LC_{50}) at the start and end of the exposure period is considered the minimum requirement. If variability is expected to be a problem, measuring test concentrations midway through the test is recommended. Note that for some test organisms (e.g. algae), static exposure systems are generally recommended because the organisms would be lost if static-renewal or flow-through systems were used. However, use of continuous flow-through exposure systems has also been investigated for algae (Grade et al., 2000).

8.1.2. Static-renewal exposure systems

170. A static-renewal exposure system is recommended where exposure can be maintained within 80-120% of nominal by renewing the test solution at ≤72-hour intervals (see Section 7.9 for special considerations for both soluble and poorly soluble MSs). Analysis of the highest and lowest test concentration and a concentration around the expected test endpoint (e.g. EC_{50}, LC_{50}) at the beginning of the test, at the end of the first (or longest) renewal cycle (before and after renewal of test solutions), and at the end of the test is considered the minimum requirement. For tests lasting longer than 7 days, measurement every week is recommended. Measuring test concentrations at the beginning and end of each renewal cycle is also recommended.

8.1.3. Flow-through exposure systems

171. Flow-through exposure systems are addressed in detail in Annex 5. The use of a flow-through exposure system is recommended where concentrations are expected to decline from nominal by more than 20% over 24 hours in static or static-renewal exposure systems. The necessary frequency of sampling should be decided upon based on the stability of the test chemical in the stock solution(s) and how often the stock solutions are renewed, such that the stability of test chemical exposure can be documented. Analytical samples should be taken at the beginning of the test to verify that the system is stable and operating correctly. If, under flow-through conditions, measured concentrations are likely to remain within 80-120% of nominal, then analysis of the highest and lowest test concentration and a concentration around the expected test endpoint (e.g. NOEC, EC_x) at the beginning, mid-point, and end of the test is considered the minimum requirement. For tests lasting longer than 7 days, measurement every week is recommended. If the concentrations are expected to decline by more than 20%, then all test concentrations should be measured, and more frequent analyses are recommended. Replicate test vessels should be measured separately. If the stock solutions feeding the diluter system are renewed during the test, they should be measured at the beginning and end of the longest renewal period. If the test chemical is stable in the stock solutions, the low, medium, and high stocks should be measured at the start of a renewal cycle at least once a week. If a metering fluctuation or malfunction is detected or observed, test concentrations should be measured.

172. Note that a static-renewal exposure system may be more suitable than a flow-through exposure system in certain instances, e.g. for testing hydrolysable test chemicals or highly biodegradable test chemicals that are hydrophobic.

8.2. SAMPLING TEST SOLUTION FOR CHEMICAL ANALYSIS

173. Sampling of test solution 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 chemical have physicochemical properties which warrant special consideration? The properties of difficult test chemicals 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? It may be desirable to choose a larger volume system to allow for improved analytical capabilities.
- 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? For example, for algae, it may be necessary to separate algae from samples prior to chemical analysis or to analyse a series of test solutions incubated under test conditions but with no algae (abiotic replicates) in order to obtain a correct determination of the dissolved test chemical concentration.
- 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? This should be determined in preliminary trials (see Section 5).

174. Sample collection and storage methods should be validated before they are applied to a definitive test. For guidance specific to MSs, please see Section 7.9.

175. If the test solution for each replicate is prepared individually, separate analysis of each replicate in a treatment level should be conducted following the sampling schedules outlined in Section 8.1. The responses in each replicate are considered independent observations and separate analysis allows the variance to be determined. Some exceptions to measuring individual replicates are listed below:

- Static exposure system. In this system, when replicate test vessels are filled from a bulk preparation, only samples from the bulk preparation for each treatment level need to be analysed at the beginning of the test and from at least one replicate at the end of the exposure.
- Static-renewal exposure system. In this system, when replicate test vessels are filled from a homogenous and reproducible bulk preparation at the beginning of the test and at renewal, only samples from the bulk preparation for each treatment level need to be analysed at the beginning of the test and at renewal. Samples should still be collected from replicate test vessels at the end of the first (or longest) renewal cycle to determine the test chemical concentration after any dissipation may have occurred (see also Section 8.1.2).
- Flow-through exposure system. In this system, when a "splitter" is used to feed more than one replicate test vessel, only samples from one replicate per treatment level need to be analysed. It is recommended to collect and store samples from all replicates in case anomalous results are found. Analysis of the stored replicates may clarify the cause and extent of the anomalous measurements. It is recommended to sample and analyse replicates receiving flow from a splitter on an alternating schedule (e.g. if there are 2 replicates [A and B], replicate A should be analysed in the first sampling time point and replicate B in the second sampling time point).
- Insufficient volume. If individual replicates contain insufficient volume to conduct the required analysis, samples for each treatment level may be pooled. Insufficient volume may occur if the analytical method requires larger sample volumes to achieve necessary detection limits. This may be more common when the test solution volume is relatively small (as in algal and daphnid studies).

- Large number of replicates. Some study designs use many replicates and it may be impractical to sample each one. In this case, if test solutions in each replicate are prepared individually, at least 2 replicates should be sampled at each sampling interval, and alternate replicates should be sampled at subsequent intervals.

9. CALCULATION AND EXPRESSION OF TEST RESULTS

176. The procedures described in Section 8 provide either nominal or measured exposure concentrations of test chemicals that are mono-component substances to be used to determine effects concentrations. Often, the exposure concentration is based on a mean measured concentration. Variability between replicates or within a given replicate over time should be minimised. Because there are different methods for calculating the mean measured concentrations, the method used should be made explicit in the reporting of test results. 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 static-renewal and flow-through exposure systems, where the measured concentrations in samples remain within 80-120% of nominal, the effect concentrations can be expressed relative to nominal or measured concentrations;
- If measured concentrations in samples do not remain within 80-120% of nominal, the effect concentration should be expressed relative to the measured concentrations. In this situation, for both flow-through and static-renewal exposure systems, effects concentrations may be determined and expressed relative to the time-weighted mean measured concentrations. Formulae for calculation of time-weighted mean measured concentrations are provided in Annex 2; and,
- For tests with chemicals that cannot be quantified by the most sensitive analytical methods at relevant concentrations, the effect concentration can be expressed based on the nominal concentrations or loading rate (for mixtures).[11] Confirming test chemical concentrations in a stock solution (if used) or at higher exposure concentrations, may provide useful supporting data. Some approaches described in this document (e.g. generator columns) necessitate that concentrations are defined with an analytical method. If concentrations cannot be quantified, direct addition approaches with defined nominal concentrations should be used to prepare testing solutions. Some fish testing guidelines (OECD TG 203, 210, 229, 230, 234, 240, 305) include validity criteria which require analytically measured test concentrations (e.g. to demonstrate that test chemical concentrations have been satisfactorily maintained within 80-120% of mean measured values). Relevant regulatory authorities should be consulted on the preferred approach in these cases.

177. It is generally recommended that all test results be expressed in terms of 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 when reported using nominal concentrations. It should also be noted that it is often useful to have both measured and nominal effect concentrations quoted. Some regulatory authorities may accept the mean initial measured concentrations to express toxicity if the initial test concentrations were below 80% of the nominal and this concentration was maintained throughout the test (within ±20 % of the initial) including the final sampling.

178. Guidance on the conventions used for expressing exposure and determining effect concentrations in tests with MSs is given in Section 7.9.

[11] For very poorly water soluble or highly hydrophobic test chemicals, a dietary exposure may be an ecologically relevant exposure route which can enable dose verification in chronic fish studies (OECD GD 171 [OECD 2012c], Fish Toxicity Testing Framework). Specific guidance for dietary exposures is beyond the scope of this guidance document but can be found in the OECD TG 305 (OECD 2012d) dietary exposure.

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ANNEX 1: COMPUTER PROGRAMS FOR PREDICTING PHYSICOCHEMICAL PROPERTIES, AND FATE, TRANSPORT AND AQUATIC TOXICITY OF TEST CHEMICAL

1. Models have been developed to estimate the physicochemical properties, fate, transport, and toxicity of chemicals. These tools may be helpful when laboratory studies or monitoring data are not available or need to be supplemented. However, the utility of predictive models is dependent on whether it has been validated. It is likely that difficult to test chemicals will also be difficult to model and particular care should be taken to ascertain whether the model is appropriate for the chemical. OECD validation principles that should be followed when using predictive software, e.g. (Q)SARs, for regulatory purposes can be found here: http://www.oecd.org/chemicalsafety/risk-assessment/validationofqsarmodels.htm.

2. Examples of computer programs are provided below. This list is not comprehensive and many other computer programs exist. Rational for why a selected model is applicable and validated for the intended use should be documented. The appropriate regulatory authority should also be consulted to ensure that the model results meet regulatory requirements.

1. Models provided by the US EPA

3. The US EPA has developed models for estimating physical/chemical properties, and fate, transport and toxicity of substances (https://www.epa.gov/tsca-screening-tools). These tools may be helpful when laboratory studies or monitoring data are not available or need to be supplemented. Information on models and tools developed by the US EPA is available at the following websites:

- For information on models and tools to assess hazard, including Ecological Structure-Activity Relationships Program (ECOSAR): https://www.epa.gov/tsca-screening-tools/using-predictive-methods-assess-hazard-under-tsca#models
- For information on models and tools to assess physicochemical properties, fate and exposure of test chemicals, including Estimation Programs Interface (EPI Suite)™: https://www.epa.gov/tsca-screening-tools/using-predictive-methods-assess-exposure-and-fate-under-tsca#fate

2. The OECD QSAR Toolbox

4. To increase the regulatory acceptance of (Q)SAR methods, the OECD is developing a QSAR Toolbox to make (Q)SAR technology readily accessible, transparent, and less demanding in terms of infrastructure costs. For additional information, please visit: http://www.oecd.org/env/ehs/risk-assessment/oecd-qsar-toolbox.htm.

3. Commercial physicochemical models

5. There are many commercially available software packages that estimate various physicochemical properties. These models may require purchase or a subscription prior to use.

- The SPARC Performs Automated Reasoning in Chemistry (SPARC) model predicts transformation rates, solvent-water partitioning, and various chemical properties (e.g. boiling point, vapor pressure, etc.) based on chemical structure. SPARC is produced by ARChem and is available at http://www.archemcalc.com/.
- ACD/Labs has developed several submodels for the ACD/Labs Percepta application that predict solvent-solvent partitioning and ADME-toxicity for a large variety of chemicals and solvent pairs. Predictions are made based on unique solvent and chemical descriptors related to hydrogen bonding, proton accepting or donating potential, and other parameters related to chemical and electronic structure. The ACD/Labs Percepta model is available at http://www.acdlabs.com/products/percepta/portal/.
- ALOGPS, produced by the VCClab, predicts the octanol-water partitioning, water solubility, and dissociation constant (pK_a) for many chemicals based on chemical structure descriptors. The model also compares results to other relevant $logP_{ow}$ models. ALOGPS is available at http://www.vcclab.org/lab/alogps/.
- The Bio-Loom program (which contains ClogP), maintained by BioByte Corp., predicts hydrophobicity and molecular refractivity parameters (e.g. $logP_{ow}$) as well as biological activity for a variety of chemicals. Bio-Loom also contains a large database of measured $logP_{ow}$, pK_a, and other chemical properties. Bio-loom is available at http://www.biobyte.com/bb/prod/bioloom.html.
- VEGA QSAR, maintained by Istituto di Ricerche Farmacologiche Mario Negri Milano, predicts biological (e.g., mutagenicity, carcinogenicity), environmental (e.g., bioaccumulation), and physicochemical (e.g., $logP_{ow}$) properties using a wide variety of QSARs. VEGA focuses on ease of use and assessment of the applicability of predictions. VEGA is available at https://www.vegahub.eu/

ANNEX 2: FORMULAE FOR CALCULATING TIME-WEIGHTED MEAN EXPOSURE CONCENTRATION

Flow-through exposure systems:

1. For flow-through exposure systems, the time-weighted arithmetic mean of the exposure concentration (where concentrations have been determined on more than two occasions during a test) may be calculated as follows:

$$C_w = \frac{\Sigma_{i=1}^{n} \frac{\left(C_{start,i} + C_{end,i}\right)}{2} w_i}{\Sigma_{i=1}^{n} w_i}$$

Where:

C_w is the time-weighted arithmetic mean concentration

n is the number of sampling periods

$C_{start,i}$ is the concentration of the fresh test solution of period i

$C_{end,i}$ is the concentration of the old solution of period i

w_i is time $t_i - t_{i-1}$, the number of hours or days in the $i\underline{th}$ interval between measurements of concentration

Static-renewal exposure systems:

2. For static-renewal exposure systems, the time-weighted arithmetic mean of a series of logarithmic means (where each logarithmic mean represents the concentration during a renewal interval) may be calculated as follows (see also Annex 6 of OECD TG 211; OECD, 2012):

$$C_w = \frac{\Sigma_{i=1}^{n} \frac{\left(C_{old,i} - C_{new,i}\right)}{\ln C_{old,i} - \ln C_{new,i}} w_i}{\Sigma_{i=1}^{n} w_i}$$

Where:

C_w is the time-weighted mean concentration

n is the number of sampling periods

$C_{new,i}$ is the concentration of the fresh test solution of period i

$C_{old,i}$ is the concentration of the old solution of period i

w_i is time $t_i - t_{i-1}$, the number of hours or days in the $i\underline{th}$ interval between measurements of concentration

Variance:

3. The variance of the time-weighted means (described above) may be expressed as follows (Madansky, 2018):

$$Var(C_w) = \frac{\sigma^2 \sum_{i=1}^{n} w_i^2}{\left(\sum_{i=1}^{n} w_i\right)^2}$$

Where:

$Var(C_w)$ is the variance of the time-weighted means

C_w can be generalised as

$$C_w = \frac{\sum_{i=1}^{n} x_i w_i}{\sum_{i=1}^{n} w_i}$$

for flow-through,

$$x_i = \frac{\left(C_{start,i} + C_{end,i}\right)}{2}$$

for static-renewal,

$$x_i = \frac{\left(C_{old,i} - C_{new,i}\right)}{\ln C_{old,i} - \ln C_{new,i}}$$

σ^2 is estimated by,

$$\sigma^2 = \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}$$

$\bar{x}$ is given as,

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

n is the number of sampling periods

w_i is time $t_i - t_{i-1}$, the number of hours or days in the *ith* interval between measurements of concentration

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ANNEX 3: TOXICITY MITIGATION TESTING FOR CATIONIC TEST CHEMICALS

1. Standard test media used for aquatic hazard testing usually has low total organic carbon (TOC <2 mg/L) content compared to surface waters, which may result in artificially high toxicity of some cationic test chemicals. Under some circumstances it may be necessary to determine the extent to which the toxicity of a test chemical is mitigated by adsorption to dissolved organic carbon (Nabholz et al., 1993; Boethling et al., 1997). Toxicity mitigation testing has been carried out with fish (US EPA, 1996), as fish are least susceptible to physical effects resulting from clogging and/or coating of gills by precipitate formed by the reaction of the cationic test chemical with the anionically charged dissolved organic carbon. However, mitigation testing has also been successfully carried out with daphnids (AFNOR, 2003; MAFF, 2000; DeSchamphelaere and Janssen, 2004; Hyne et al., 2005), green algae (Costa et al., 2014; Stevenson et al., 2013), and bacteria (Chen et al., 2011). Some regulatory authorities may prefer that these test organisms be used in mitigation assessment in the interest of animal welfare. The relevant regulatory authority should be consulted before initiating testing to define the preferred approach to meet regulatory requirements.

2. If a cationic test chemical has been determined to be intrinsically toxic in test medium containing <2 mg/L TOC, the test chemical should be tested in test media prepared with at least two different concentrations of dissolved humic acid. The first of these tests should be carried out at a humic acid concentration typically not exceeding 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. Floc formation should be evaluated in a preliminary investigation at all expected treatment levels and over the test duration since it can be time dependent and can vary based on polymer concentration, TOC and other water chemistry parameters. Static-renewal tests may be needed if floc formation is problematic. The test organisms should be acclimated to the final dilution waters containing added humic acid.

3. The concentration of total organic carbon should be measured in the control test medium of each test (e.g. without added humic acid, the test with high [~20 mg/L] humic acid and the test with intermediate [10 mg/L] humic acid). TOC samples should be taken from the controls at the beginning of the toxicity tests and in new control test medium used for renewal (if applicable). For each test, the TOC measurement of control test medium should be reported separately from test chemicals, and a mean TOC value from the fresh control test medium should be reported as the overall TOC value for the test. TOC measurements in aged control test medium may also be informative; however, since the TOC value will be influenced by biological exudates these should not be used to evaluate the overall control test medium TOC for the test. Measuring TOC in standard test medium may be challenging due to the generally low sensitivity of the method (~1 mg/L). The accuracy of the analytical method used for TOC measurement should be verified and reported. Acidifying samples prior to analysis will likely cause soluble humic acid to precipitate, so at least three individual measurements should be performed on each TOC sample and the mean reported as the sample TOC value.

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

Information on the mitigating effects of dissolved organic carbon can be important supplemental information when assessing the hazard of a test chemical to the environment, and should be provided when applicable and available. Regulatory agencies (e.g. US EPA, 2013; US EPA, 1996; MAFF, 2000) have suggested that this information would be taken into account when evaluating test chemicals.

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ANNEX 4: TOXICITY MITIGATION TESTING WITH ALGAE FOR TEST CHEMICALS WHICH FORM COMPLEXES WITH AND/OR CHELATE POLYVALENT METALS

1. The purpose of this annex is to supplement Section 7.5.2 and provide additional guidance for algae tests with test chemicals that may form complexes with and/or chelate polyvalent metals. The outcome of this mitigation testing should assist in distinguishing between inherent test chemical toxicity and secondary effects resulting from complexation. Examples of such chemicals are anionic polymers in the poly(carboxylic acids) class and polyanionic monomers (Nabholz et al. 1993; Boethling et al., 1997). Overchelation of the nutrients needed for algal growth by these types of test chemicals in the standard algal growth test medium manifests in reduced growth, which is simply due to lack of availability of nutrients and not toxicity. Most surface waters have hardness well above the hardness of standard algal growth test medium. The requirements for algal toxicity testing in relation to risk assessment for test chemicals 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 to assess effects in more typical receiving waters:

a. Test the chemical as it is in standard algal growth test medium with a hardness in the range 15 to 24 mg/L as $CaCO_3$;

b. test the chemical as it is in modified algal growth test medium with a hardness of approximately 150 mg/L as $CaCO_3$;

c. test the chemical as the Ca salt in standard algal test medium by adding an equivalent amount of Ca^{2+} to the stock solution (assuming the chemical is a Na or K salt); and

d. test the chemical as the Ca salt in modified algal test medium as for (b) above.

If a significant difference in toxicity is noted between the two algal media hardness levels, additional testing could be performed at a hardness equivalent to that in the expected receiving waters.

2. Testing a chemical 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 test chemical might be to add 1 g active ingredient of the test chemical 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 solution. 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 solution and should not be removed by filtration or centrifugation.

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ANNEX 5: FLOW-THROUGH EXPOSURE SYSTEMS

1. Flow-through exposure systems are used in aquatic toxicity tests for two major reasons: to maintain water quality and to maintain stable test concentrations throughout a test. They are typically used in longer term bioconcentration, early life stage, fish full lifecycle, or multigeneration tests in order to maintain appropriate water quality conditions (e.g. dissolved oxygen, ammonia, pH, etc.) and to support the increased biomass associated with such tests. The increased biomass and organic waste resulting from the number, size, and growth of organisms in such tests directly impact water quality, making static systems inadequate and static-renewal systems impractical for such tests. An example of a flow-through exposure system is presented in Figure 1.

2. Flow-through exposure systems are also used in both short term and long term tests when the physicochemical properties of the test chemical result in unstable test concentrations in static and static-renewal exposure systems. The major problem is loss of test chemical from processes including biodegradation, oxidation, hydrolysis, photolysis, volatilisation, or adsorption to materials that make up the exposure system. A flow-through exposure system can alleviate the problem of unstable concentrations by continually adding new test solutions to test tanks, although they are also limited by the chemical properties mentioned above.

3. The amount of test solution added to a tank is typically expressed as the number of tank volume turnovers in a 24-hour period. The number of turnovers in the flow-through exposure system should be guided by both water quality and the stability of the test chemical, but typically ranges from 5 to 15 turnovers per day. Flow rates of test medium (i.e. dilution water), stock and test solutions should be monitored regularly in order to maintain constant exposure concentrations. In cases where a change in test chemical concentration is observed over the course of the study, adjustments to stock and test solution delivery can be made. In longer term tests in which biofilms develop flow rates may need to be increased to maintain dissolved oxygen concentrations. However, flow rates should not be so high as to have an adverse impact on the test organism (e.g. affecting temperature regulation or leading to reduction of food availability).

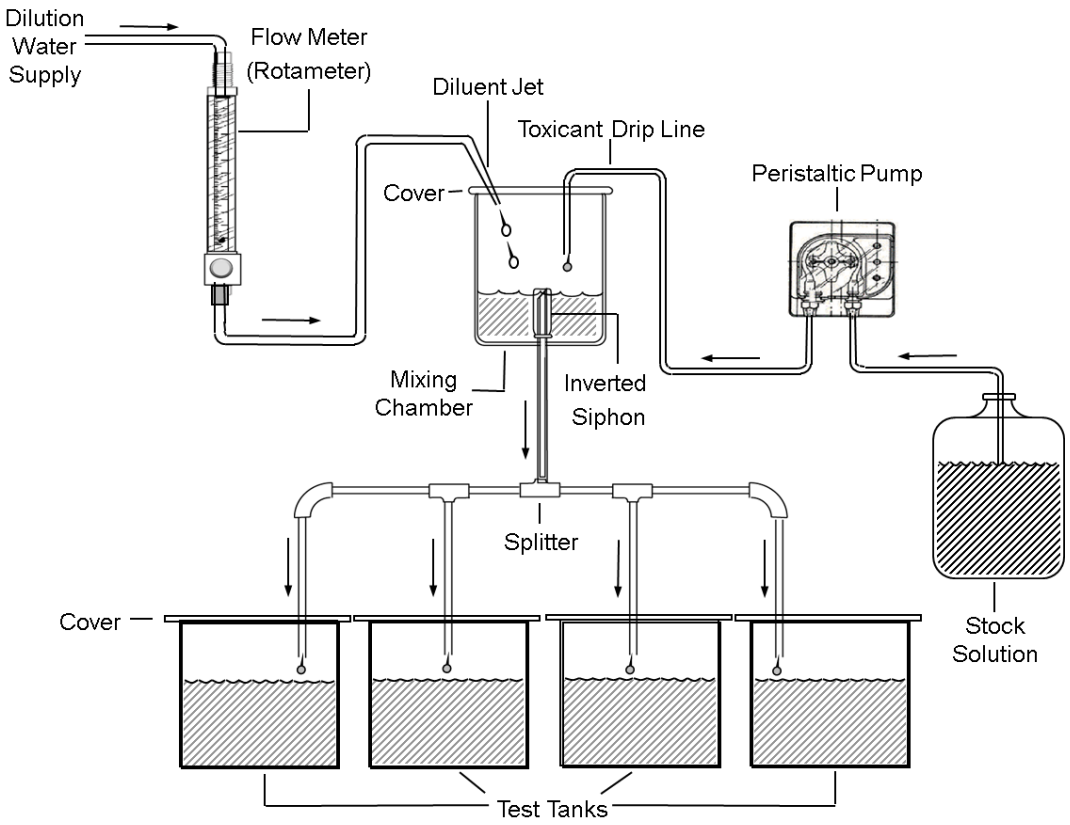
4. Prior to introducing test organisms, the flow-through exposure system should be calibrated until there are consistent flow rates between replicates and guideline requirements are met (see for example Figure 1). Prior to the start of a test, samples of test solutions should be collected and analysed to confirm test concentrations and to insure proper delivery of stock solutions into test tanks or beakers. Many of the problems of difficult test chemicals can be overcome in flow-through exposure systems by adding test solutions to the replicate tank or beaker faster than the test chemical is degrading or lost from the test replicate (this should be confirmed through analytical measurements during the equilibration pre-test period as well as during the exposure). However, a flow-through exposure system does have limits when rapid degradation, adsorption, or volatility are extreme.

5. As previously mentioned, physicochemical properties of the test chemical are important factors that need to be considered when preparing stock solutions for flow-through exposure systems. Ideally the stock solution can be prepared directly in the test medium at a concentration greater than the highest exposure concentration and it is preferable that the highest test concentration should be at least 100 to 1000 times below the

water solubility limit of the test chemical. This allows for the preparation of a relatively smaller volume of concentrated stock solution that can easily be diluted into the range of test concentrations being tested. If this is not the case, large volumes of stock solutions will need to be prepared and in cases where the test chemical is not toxic at the saturation concentration, the volume of stock required (and therefore the amount of test chemical required) may be a potential limiting factor for testing. Before starting an experiment, stability of the stock solutions should be known to ensure an appropriate schedule for preparation. With stable test chemicals, prolonged mixing may be used for preparation provided that an emulsion is not created.

6. Note that for some test organisms (e.g. algae), static tests are generally recommended because the organisms would be lost if renewal or continuous flow-through systems were used. However, use of continuous flow-through exposure systems has also been investigated for algae (Grade et al., 2000).

Figure 1. An example of a flow-through exposure system (courtesy of EAG Laboratories)



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ANNEX 6: PASSIVE DOSING

1. Passive dosing is a technique for establishing and maintaining freely dissolved concentrations of poorly soluble test chemicals (i.e. hydrophobic organic chemicals) including MSs, such as UVCBs, in aquatic toxicity tests. A biocompatible polymer is first loaded with the test chemical and then included in the test system where it acts as a partitioning donor that controls exposure concentrations throughout the test (Mayer et al., 1999; Smith et al., 2010a; Butler et al., 2013). Passive dosing has previously been applied to single chemicals as well as simple mixtures and UVCBs (Smith et al., 2013a; Butler et al., 2016; Redman et al., 2017).

2. Over the past two decades, a range of loading principles and techniques have been developed for the passive dosing application. Below are a few of the more commonly practiced passive dosing techniques relying on the exploitation of the physicochemical properties of hydrophobic organic compounds:

a. The polymer can be saturated with the test chemical, by placing it in a suspension of the test chemical in methanol[12] (Smith et al., 2010b) or by direct immersion into a liquid test chemical (Stibany et al, 2017). Passive dosing from such a saturated polymer will then provide exposure at or near the saturation concentration (Smith et al., 2010a). Further, direct syringe injection of a single test chemical or UVCB into medical grade silicone tubing offers the versatility to perform limit exposure at the saturation concentration or as a dose response by varying the volume/mass (Redman et al., 2017). This technique also allows for limit concentration, water saturation exposures for solids by creating a suspension in silicone oil and then injecting that suspension into a length of silicone tubing.

b. The polymer can be loaded to a certain concentration ($C_{polymer}$) by equilibrium partitioning from methanol solutions of the test chemical, and this can be done in many different ways (see e.g. Smith et al., 2010a, Butler et al., 2013, Seiler et al., 2014, Vergauwen et al., 2015, Butler et al., 2016). The freely dissolved concentration of the test chemical in the test will then be given by $C_{polymer}$ divided by the polymer to water partition constant. This partition constant can be determined in a separate experiment or as part of the toxicity experiment in controls free of test organisms. When working at the saturation concentration, the relationship between $C_{polymer}$ and $C_{methanol}$ does not need to be determined. It is also possible to prepare fractions of saturation (e.g. S; S/2; S/4 etc.), without knowing the partition ratios. However, in order to relate toxicity to an absolute concentration, the concentration in the test needs to be measured.

c. The polymer can also be loaded by first adding a small volume of methanol spiking solution and then sequentially adding water in order to push all test chemical into the polymer (Birch et al., 2010). When the test chemical is close to the saturation

[12] Most solvents other than methanol will lead to substantial swelling of the polymer and the solvents will then also leak out during the test. Methanol is the most prevalent solvent used in the current published literature. Other solvents might work, but this requires substantial experimental validation work.

concentration, care should be taken not to cause emulsions/turbidity if the saturation concentration is exceeded. In this case, $C_{polymer}$ is defined by the mass of test chemical in the methanol stock and the mass in the polymer. For MSs special attention is required in order to achieve the desired mixture composition and level (Rojo-Nieto et al., 2012).

3. Once the loading is complete, the polymer is washed with de-ionised water and dried to ensure that all solvent is removed before adding it to the test vessels (in the case of volatile test chemicals, methanol is removed by washing only). For very volatile test chemicals, avoid volatilisation of loaded test chemical from polymer prior to deployment by storing the loaded polymer in de-ionised water if it is not immediately deployed to the test medium. After adding the loaded polymer to the test medium, a concentration of the test chemical is achieved via equilibrium partitioning between the polymer and the test medium. The time to equilibrium between $C_{polymer}$ and the test solution is dependent on mixing kinetics (Smith et al., 2010a). Losses of test chemical from the test solution during the test due to sorption, volatilisation, degradation, uptake or transformation by organisms are compensated for by additional release from the polymer (Butler et al., 2013; 2016), which allows constant freely dissolved concentrations of test chemicals to be maintained while avoiding the use of a solvent. As with all aquatic toxicity tests, exposure should be confirmed by measuring the test chemical concentration in the test solution during or after incubation using a suitable analytical method (see Section 7.9 for MSs).

4. Furthermore, it may be appropriate to determine the amount of test chemical remaining in the polymer at the end of the study (Smith et al., 2010a; 2013a; 2013b). Concentrations of chemicals in the polymer will generally be stable for extended periods of time (e.g. weeks and months) if the test chemical is highly hydrophobic (e.g. $LogP_{ow}$ >5), since in this case the polymer has a very high buffer capacity relative to water. It is worth emphasising that passive dosing controls the freely dissolved concentrations of the test chemical and that binding of test chemical to test solution constituents (e.g. food residues, feces, algal cells and algal exudates) can lead to increases of the total concentration that includes bound and unbound molecules (Gouliarmou et al., 2012). Such an increase of total concentrations during the test should not disqualify a study but rather be seen as a confirmation that the passive dosing technique as intended buffered the freely dissolved concentration and was necessary.

5. The polymer should be of food, medical or analytical grade in order to minimise the possibility for impurities leaching from the polymer and causing or interfering with the toxicity of the test chemical. PDMS is particularly suited, since it combines excellent partitioning properties with high diffusivities for most hydrophobic organic compounds. Several polymer geometries have been used for passive dosing. Ring and rod-shaped polymers are characterised by a large surface area and a large polymer volume, which leads to fast passive dosing kinetics and a high buffer capacity for dosing, i.e. the polymer acts as a reservoir of the test chemical which compensates for the test chemical lost from the system. Silicone can also be cast into the bottom of test jars or sheets placed in test wells on plates (Kramer et al., 2010; Slootweg et al., 2015; Smith et al., 2010b). Further, test chemical injected into silicone tubing has been shown as a successful application of passive dosing (Redman et al., 2017).

6. When using passive dosing in toxicity testing it should be ensured that the presence of the polymer is not causing an effect on the organism or that it is biasing the observation of this effect. This is first of all ensured by using a high grade polymer to start with and then to clean them thoroughly by placing them into solvents that are compatible with the

specific polymer. It is advisable that the polymer used in the control is exposed to a pure solution of the solvent used for loading, e.g. methanol.

7. Passive dosing is ideal for aquatic toxicity or bioconcentration testing in relatively small vessels with many replicates, e.g. well plates (Smith et al., 2010a). Hence, passive dosing is explicitly useful for testing of algae, invertebrates, early life stages of fish and small adult fish. Passive dosing can be applied in the fish embryo acute toxicity test (Butler et al., 2013; Seiler et al., 2014; Vergauwen et al., 2015; Butler et al., 2016). Applicability of passive dosing in the fish early-life stage test has been demonstrated by Butler et al. (2013) maintaining concentrations of phenanthrene in a flow-through exposure system over 30 days. Further, Butler et al. (2016) employed a passive dosing approach using silicone O-rings to generate and maintain aqueous concentrations of single chemicals as well as a 10-compound simple mixture during a 30-day fish early life stage experiment. Similarly, passive dosing has also been used in a flow-through setting that adequately maintained concentrations over 8 days for hydrophobic test chemicals at a flow of 100 mL per minute (Adolfson-Erici et al., 2012). Additional work is needed to fully optimise passive dosing in flow-through systems at a larger scale. Since passive dosing is very effective at testing test chemicals at their saturation concentration, this technique can be used to effectively test chemicals using a limit test (Smith et al., 2010a, Stibany et al., 2017). Testing exactly at the saturation level compared to using spiked nominal concentrations can reduce the number of test replicates required for robust data and save resources and test animals (Seiler et al., 2014). Passive dosing can also be applied to the investigation of partitioning of complex environmental sediment contaminations in the sediment-water-biota system (Bandow et al., 2009a, 2009b).

8. Passive dosing has certain limitations. It is susceptible to depletion of the test chemical in the polymer. This typically becomes an issue with test chemicals that are more water soluble or have a lower $\log K_{ow}$. Further, it largely is limited to chemicals with high polymer to water partition constants, which basically sets the buffer capacity of the approach (i.e. how many times the aqueous concentration can be replenished by partitioning from the passive dosing polymer). As a general rule, passive dosing works well for chemicals that are hydrophobic and lipophilic and have $\log P_{ow}$ values >3. Additionally, a disadvantage of the approach can be the additional work load of cleaning, loading and applying the passive dosing polymers. Finally, degradation of test chemicals can potentially lead to the formation of toxic and better soluble degradation products in the aqueous test solution, which may exert adverse effects at longer test duration.

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