
ECETOC

Monograph No. 26

Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstable Substances

September 1996

Monograph No. 26

Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstable Substances

September 1996

ISSN-0773-6047-26

Brussels, September 1996

©ECETOC copyright 1996

Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstable Substances

CONTENTS

SUMMARY	1
1. INTRODUCTION	3
2. BACKGROUND	4
2.1 GUIDELINES FOR AQUATIC TOXICITY TESTS.....	4
2.2 DEFINITION OF A SUBSTANCE.....	5
2.3 LEGISLATION.....	5
2.4 USE OF AQUATIC TOXICITY DATA.....	7
3. SUBSTANCES REQUIRING SPECIAL CONSIDERATION FOR TESTING PROCEDURES	8
3.1 LOW SOLUBILITY.....	9
3.1.1 True solutions.....	10
3.1.2 Aggregated Systems.....	10
3.1.3 Colloidal Dispersions.....	11
3.2 ADSORPTION.....	11
3.3 HIGH VOLATILITY.....	11
3.3.1 Henry's Constant.....	12
3.3.2 Vapour Pressure.....	12
3.4 INSTABILITY.....	12
3.4.1 Hydrolytic and Oxidative Stability.....	12
3.4.2 Photodegradation.....	12
3.4.3 Biodegradation.....	13
3.5 COMPLEX SUBSTANCES.....	13
4. USE OF AUXILIARY AGENTS IN TOXICITY TESTS	14
4.1 EFFECTS ON SOLUBILITY IN THE TEST MEDIUM.....	14
4.1.1 Solvents.....	14
4.1.2 Surfactants.....	16
4.2 EFFECTS ON TOXICITY OF TEST SUBSTANCE.....	17
4.3 CONCLUSIONS.....	18
5. REVIEW OF UPTAKE MECHANISMS AND THEIR SIGNIFICANCE FOR AQUATIC TOXICITY	19

5.1 AQUATIC TOXICITY	19
5.2 UPTAKE ROUTES	20
5.3 FACTORS INFLUENCING UPTAKE.....	21
5.3.1 Duration of Exposure	21
5.3.2 Bioavailability	22
5.4 CONCLUSIONS	23
6. POSSIBLE EXPLANATIONS FOR ADVERSE EFFECTS ABOVE THE LIMIT OF WATER	
SOLUBILITY.....	24
6.1 EFFECTS OF MINOR COMPONENTS	24
6.2 EFFECTS OF THE INSTABILITY OF THE TEST SUBSTANCE.....	24
6.3 PHYSICAL EFFECTS.....	25
6.4 EFFECTS OF LIGHT ATTENUATION	25
6.5 EFFECTS OF PARTICULATE UPTAKE	26
6.6 CONCLUSIONS	27
7. STRATEGY FOR TESTING AND INTERPRETATION OF TEST RESULTS	28
7.1 PERFORMANCE OF TEST.....	28
7.1.1 Stability assessment	29
7.1.2 Media preparation and use of auxiliary agents.....	29
7.1.3 Test Design.....	32
7.1.4 Analytical support.....	36
7.1.5 Calculation and Expression of Results	41
7.2 INTERPRETATION AND USE OF DATA.....	42
7.2.1 Use of Data for Hazard Identification and Classification and Labelling.....	42
7.2.2 Use of data for risk assessment	43
7.3 FUTURE RESEARCH	44
8. SUMMARY OF RECOMMENDATIONS.....	46
APPENDIX A. MEASUREMENT AND CALCULATION OF SOME PHYSICOCHEMICAL PROPERTIES	
AND THEIR LIMITATIONS RELEVANT TO ECOTOXICITY TESTING.....	48
A.1 SOLUBILITY OF SUBSTANCES IN WATER	48
A.2 MEASUREMENT OF ACID DISSOCIATION CONSTANT	51
A.3 MEASUREMENT OF HYDROLYTIC, OXIDATION AND PHOTODEGRADATION RATES....	51
A.4 MEASUREMENT OF OCTANOL/WATER PARTITION COEFFICIENT	51
A.5 MEASUREMENT OF VAPOUR PRESSURE AND VOLATILISATION	51

APPENDIX B. DEFINITION AND ANALYSIS OF COMPLEX SUBSTANCES	53
APPENDIX C. WATER ACCOMMODATED FRACTIONS.....	55
APPENDIX D. MECHANISMS FOR THE TRANSPORT OF SUBSTANCES ACROSS MEMBRANES .	57
BIBLIOGRAPHY.....	59
MEMBERS OF THE TASK FORCE	65
MEMBERS OF THE SCIENTIFIC COMMITTEE.....	66

SUMMARY

The objective of the Task Force was to review the scientific validity for the application of standard aquatic toxicity tests to substances that are difficult to test, and to develop guidance for the performance of tests and interpretation of results from such studies.

The report discusses the types of substances requiring special consideration for testing procedures, namely substances with attributes such as low solubility, adsorption, high volatility, instability, light attenuation (colour) and complexity, i.e. multicomponent substances.

To facilitate the optimal performance of tests and interpretation of the data on difficult-to-test substances, it is particularly important to have information on the composition and identity of the test substance, as well as appropriate physico-chemical data, prior to performing any tests. Thus, the likely behaviour of the substance(s) may be predicted and the appropriate experimental design employed.

The report considers the effects of dispersants and co-solvents as aids to preparation of test media; this review is made in the context of a discussion of the modes of uptake of compounds by test organisms. The driving force for uptake is the dissolved concentration in the aqueous medium. The presence of undissolved substance does not influence the relationship between dissolved concentration and uptake. Hence, there is no advantage in testing above the water solubility limit in order to assess the inherent toxicity. This conclusion contradicts some regulatory guidelines and some reports of dose-response relationships of inherent toxicity above the solubility limit. Possible explanations of such responses are provided.

Since regulatory guidelines and methods for aquatic toxicity testing were developed for essentially pure substances that are soluble and stable in water, they consequently require modification to properly account for substances which are difficult to test. In this report, good practice for testing is reviewed, including sampling and the extent of analytical chemistry that is required, and guidance is provided on the use and interpretation of the results.

The information and arguments developed are brought together in a suggested strategy for testing, centred around a flow scheme which covers all substance types, including those that are not difficult to test. The scheme describes the limits on the interpretation of test results that apply to risk assessment.

Correct interpretation of effects observed in aquatic toxicity tests is dependent upon:

- test media being prepared in a manner that is appropriate to the objectives of the study and the nature of the test substance;
- correct measurement and expression of exposure levels;
- the distinction of true toxicity from indirect physical effects of the substance, which should be avoided.

With regard to EU legislation, the following recommendations are made:

Risk assessment

In an initial risk assessment for substances that are not acutely toxic at their water solubility limit, the following conservative approach is proposed in order to avoid unnecessary chronic testing: The highest measured soluble concentration (or solubility limit) is taken as the acute LC₅₀ and the assessment factor usually applied to the LC₅₀ is assigned to that concentration in order to calculate a safe upper limit for a PNEC.

Classification and labelling

If sparingly water soluble substances are toxic at a concentration below their water solubility then they should be classified according to the Directive. However, for sparingly soluble substances which in acute tests do not display toxicity at the water solubility limit and toxic impurities are not present in significant amounts, the toxicity criterion for classifying a substance as “dangerous to the environment” is not fulfilled.

Additional specific recommendations and findings discussed and developed by the Task Force are summarised in Section 8.

1. INTRODUCTION

Laboratory tests are carried out on chemical substances in order to assess their toxicity to aquatic organisms. Test methods for obtaining the data have been described by various organisations including the OECD, US-EPA, EU and ISO. The methods are typically designed for substances which fit the generic description of being essentially pure, readily water soluble, chemically stable and non-volatile. Methods for testing substances which do not meet this description have not been defined. As a consequence, when test methods for soluble, stable and non-volatile substances are applied to sparingly soluble, unstable or volatile substances or those of a complex composition, difficulties are found in two distinct aspects of the assessment of toxicity:

- maintaining constant and bioavailable concentrations;
- interpreting the results obtained.

Maintenance of exposure concentrations is important since effects should be interpreted as being due to the exposure concentrations of test substance present in the test medium. If the concentration drops significantly, or the test substance is present in a biologically unavailable form, estimation of the true exposure concentration is difficult. Without knowledge of the true exposure the interpretation of the observed effects and their extrapolation to possible environmental effects is compromised.

Against this background ECETOC established a Task Force with the following terms of reference:

- review the scientific validity for the application of standard aquatic toxicity tests to substances at levels exceeding their solubility where the biological effects do not occur at the limit of solubility and to those substances that are difficult to maintain at constant concentrations;
- develop guidance for the performance of tests and interpretation of results from such studies;
- appraise the influence of auxiliary agents (solvents, surfactants) on the distribution of the test substance in water and the subsequent ecotoxic evaluation.

The literature was reviewed and considered in establishing a technically based position on these issues. Some recommendations are made in the report with a view to contributing to the establishment of standard approaches and procedures for testing sparingly soluble, volatile, unstable and/or complex substances.

Testing of preparations (e.g. crop protection formulations or consumer products), metals and metal compounds (see OECD, 1995) and effluents is outside of the scope of this report. Some of the recommendations of this report may, however, be suitable for testing preparations.

2. BACKGROUND

2.1 GUIDELINES FOR AQUATIC TOXICITY TESTS

The data derived from the standard aquatic toxicity tests are used for two primary purposes:

- for hazard identification and classification of the substance in compliance with regulatory criteria;
- for establishing a maximum concentration not expected to cause adverse effects to aquatic life, as part of risk assessment.

Guidelines for conducting aquatic toxicity tests have been published by various national regulatory agencies and international organisations. Within the EU the most widely accepted testing guidance has been published by the EEC (1992) and OECD (1992). In the USA corresponding guidelines were published by the US-EPA (1985). Some of the most frequently used tests for determining aquatic toxicity are:

- Activated sludge respiration inhibition test
- Algal (72h) growth inhibition test
- Daphnia (48h) acute test
- Fish (96h) acute test
- Daphnia (21d) reproduction test
- Fish chronic tests: prolonged toxicity; fish, early life stage toxicity test.

The guidelines were initially developed with the aim of evaluating the toxicity of substances that can be considered readily water-soluble and stable (OECD, 1992). Many substances fall outside these criteria and yet the requirements for test data remain. It is for these substances that additional guidance on testing and data interpretation is required.

Testing of substances which are sparingly water soluble, volatile or chemically unstable present difficulties in the execution of standard toxicity tests and in the interpretation of their results for the two purposes mentioned above.

2.2 DEFINITION OF A SUBSTANCE

A chemical compound is an entity to which usually a single structural formula can be assigned. In practice, commercial chemicals, even if they are named according to the compound being the main component, contain other compounds as impurities. Recognising this, the EU definition of a substance is given in the EU Directive 67/568/EEC relating to the classification, packaging and labelling of dangerous substances as:

"chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the products and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition."

This report uses the word 'substance' according to the above definition. The word 'component' is used to designate a constitutive compound present in a defined substance. 'Test substance' is used for the substance taken for carrying out a laboratory test which may be the commercial substance as such or, in special cases, one or more of its components in a purified form or a degradation product.

For the purposes of testing, it is possible to consider two distinct cases with regard to the composition of commercial substances:

- (a) a substance with one major component containing minor components as impurities. Such a substance is called "essentially pure" in this report; however, the impurities may still be of significance;
- (b) a "complex substance" which is a homogeneous aggregate of a number of compounds with different physical and/or chemical properties, which can be separated by physical means. Such commercially produced complex substances are also listed in EINECS¹ or ELINCS². Typically, this description includes oil products, mixtures of homologues or isomers, reaction products made from impure starting materials or mixtures of substances and stabilisers. Within the EU definition, this is different from a "preparation" which is a deliberate mixture of substances for purposes other than just stabilisation.

2.3 LEGISLATION

¹ European Inventory of Existing Chemical Substances

Ecotoxicity data on substances are required from legislators in many countries and from supranational organisations for various purposes. For example, at the international level, PARCOM (1990) (now known as OSPAR) has proposed the testing of marine species for evaluating the risk of chemicals released into the North Sea. In addition, some national authorities have developed schemes for evaluating the risks of "existing" and "new" substances (summary table in ECETOC, 1993a).

Within the EU, the most important legislation for chemical substances are the Seventh Amendment of Directive 67/548/EEC (EEC, 1992) and the Existing Chemicals Regulation (EEC, 1993a).

Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of substances was first designed to protect man from hazards arising from handling, transport and storage of "dangerous substances", i.e. those which fall under the criteria which trigger classification. It provides relevant information in the form of 'risk-' and 'safety-phrases'. It was amended several times, and its 7th Amendment (EEC, 1992) includes a symbol for the classification "dangerous for the environment", prescribes notification procedures for new substances and requires a risk assessment to be performed for these substances. The classification scheme for the aquatic environment is based along with other information on an upper limit for acute toxicity of 100 mg/l.

Council Regulation 793/93 of 23 March 1993 requires a risk assessment to be performed for existing substances (EEC, 1993a) in a similar manner as for new substances.

Environmental risk assessment in both cases consists of a comparison of the Predicted (or actual) Environmental Concentration (PEC) with the Predicted No Effect Concentration (PNEC), so that a PEC/PNEC ratio may be derived. Both the assessment of the intrinsic hazardous properties of the substances and the assessment of the environmental concentration are based on a tiered test approach which allows the PEC and the PNEC to be improved independently from each other by using better data for their determination (ECETOC, 1993a).

The PEC can be determined by mathematical modelling of the distribution and fate of the substance in the environment or by environmental monitoring. Modelling takes into account such factors as the intended use of the substance, its potential discharge into the environment, degradation, volatility, solubility and sorptive properties.

The PNEC is normally calculated by extrapolating from the results of laboratory toxicity tests by the use of assessment factors. The assessment factors are conservative, reflecting the uncertainty that exists in extrapolation. It is therefore critical that the data used for the estimation are appropriate, since

² European List of Notified Chemical Substances

inadequacies will be compounded by the assessment factors. For new substances the type and extent of testing being determined by the quantity of substance that is manufactured or placed on the market within the EU.

Detailed guidance and review of the process of environmental risk assessment for "new" and "existing" substances has been provided by ECETOC (1992; 1993a; 1994a; 1994b; 1996) and was published recently by the EU (EEC, 1993b; 1994).

In the case of substances that are difficult to test due to their low water solubility, high volatility, instability or the tendency to adsorb to surfaces, the extrapolation procedure from toxicity data to a PNEC potentially bears an additional number of uncertainties.

2.4 USE OF AQUATIC TOXICITY DATA

The two main purposes for the use of aquatic toxicity data, i.e. classification and labelling on the one side and risk assessment on the other, require different considerations concerning any potential exposure situation.

Classification and labelling addresses proper handling of the substance in order to prevent any undue direct exposure of man and environmental organisms. Therefore, aquatic toxicity data will have to be established for the commercial substance as such.

In environmental risk assessment, however, releases at all stages of the life cycle of a substance have to be taken into account. Release estimation is then followed by fate modelling, i.e. mitigating measures, distribution between compartments, degradation, adsorption. Continuous exposure may have to be distinguished from intermittent exposure. These complex processes may have serious consequences in the higher tiers of risk assessment for substances which are difficult to test. Whereas 'instability', 'volatility' and 'adsorption' are taken into consideration in modelling a PEC, difficulties arise particularly for sparingly soluble substances with toxicologically significant impurities and for complex substances. Depending on the physical and chemical properties of the individual components these may or may not be separated in the fate process to a significant extent. This may have consequences for the testing strategy (selection of the right component(s) for testing) and for the interpretation of the results in the higher tiers of the risk assessment process.

3. SUBSTANCES REQUIRING SPECIAL CONSIDERATION FOR TESTING PROCEDURES

Substances which are sparingly water soluble, volatile or chemically unstable present difficulties in the execution and interpretation of standard toxicity tests. Specifically, those attributes of substances which may complicate test conduct and/or data interpretation include low solubility, adsorption, high volatility, instability, and heterogeneity, e.g. multicomponent substances. Key conceptual and technical questions which need to be addressed when deciding on testing procedures for different types of substances are summarised in Table 1. Additional technical questions related to coloured substances are described in section 7.1.3.

Substances of low solubility may present additional difficulties, such as they may appear to express concentration related toxicity above the water solubility limit. This is not consistent with the concept that toxicity is a function of the soluble concentration.

For adsorptive, volatile, and unstable substances, the issues are:

- how to maintain the exposure concentration, and
- how to ascribe the effects of unstable substances (to the parent substance, the breakdown products or the mixture).

Complex substances provide difficulties in conducting and interpreting toxicity studies, as they can contain many components, each with one or more of the attributes of substances which are difficult to test. Complex substances frequently give test solutions which have a composition that is quite different to that of the substance itself.

Prior to conducting ecotoxicological tests, appropriate physico-chemical characterisation of the substance is required. These requirements are generally stipulated in the test guidelines. The data are needed to understand potential problems with testing and interpretation of results. It is important to note that impurities frequently have markedly different properties from the main component of a substance. If there are indications that their properties may significantly influence the observed toxic effects of the test substance, this should be considered for possible improvements of the test design and in the interpretation of the results in the context of environmental risk assessment (see Section 2). The various procedures for determining physico-chemical properties are reviewed in Appendix A in the context of difficult-to-test substances.

Table 1: Key Conceptual and Technical Questions for Difficult-to-test Substances

Attributes	Conceptual Questions	Technical Questions	Examples grouped by chemical categories
Low solubility may adsorb onto surfaces; sometimes mixtures	Is it appropriate to test above limit of water solubility? How are effects observed above the solubility limit to be interpreted?	How to prepare true test solutions? How to create and maintain dispersions or emulsions?	Oils, insoluble polymers, pigments, organometallics, lubricant additives, petrochemicals and solvents, many crop protection active ingredients and pharmaceuticals, inorganics
Adsorption	Should steps be taken to maintain exposure concentrations?	How to minimise losses of the test substance?	see solubility (above)
High volatility often coupled with low solubility	Should steps be taken to maintain exposure concentrations?	How to minimise losses of the test substance?	Oils, fine chemicals, petrochemicals and solvents
Instability hydrolysis or oxidation in water, photo- or biodegradation	Should steps be taken to maintain exposure concentrations? Is it appropriate to determine the toxicity of the mixture of parent compound and breakdown products or of the individual breakdown products?	How to minimise losses of the test substance? What to analyse for?	Organic substances with reactive functional groups, organometallics, metals/carbon blacks, monomers, reactive dyes, certain crop protection active ingredients
Complex substances many components, often presenting analytical difficulties	What is the upper "concentration" limit for testing? How to express the test results?	How to prepare test media? What to analyse for?	Soluble polymers, insoluble polymers, lubricant additives, surfactants

3.1 LOW SOLUBILITY

For this report, 'sparingly soluble' means solubility limits < 100 mg/l in water, and 'very low solubility' means solubility limits < 1 mg/l in water. The value of 100 mg/l was chosen because this is the limit for toxicity testing as described in the 7th Amendment (EEC, 1992).

It is essential to note that solubility in water may differ from that in the medium used under the conditions of standard tests. For example, the presence of dissolved salts and organic material in the test medium can have significant effects on solubility. For ionisable substances the pH will often have a decisive influence on solubility. In some cases it would be essential to know the solubility in the test medium. It is

important to note, however, that the practically achievable concentration in the test medium may not be as high as that measured in smaller scale physico-chemical tests.

3.1.1 True solutions

The solubility of a substance in water is defined as the maximum concentration which can be reached under specified conditions of temperature and pressure, where a homogeneous single phase exists. As soon as this concentration is exceeded, a different phase is formed which is often dispersed in the solution.

For a single component substance, i.e. a pure substance, solubility is the amount of the substance that dissolves when an excess of the substance is present, at equilibrium. Super-saturation can be induced, for example, by the effects of temperature variation. For many substances, where the water solubility is very low, a value for the water solubility may be difficult to determine. Results may vary depending on the method used for the determination.

For complex substances, the concept of a single defined water solubility has no meaning, since the total amount in solution will be the equilibrium amount of all dissolved components, which may be different from the composition of the complex substance itself and will vary depending on the amount of substance added, i.e. the loading rate (see also Appendix B). Therefore the 'water accommodated fraction' (WAF) approach is of use.

3.1.2 Aggregated Systems

At a macroscopic scale, a test liquid may appear to be a single phase, but investigation at a molecular scale may show this not to be the case. For example, the formation of micelles by molecules displaying both hydrophilic and lipophilic properties has been described in the literature (e.g. Rosen, 1976). There are other aggregated structures that can form in water, such as vesicles and microemulsions, which are also of dimensions below the range of characterisation by normal light scattering techniques.

At a sufficiently high concentration, a surface-active agent introduced into a test medium partitions between the dissolved phase, a micellar phase, a phase adsorbed on available surfaces (the biological material included) and the air interface. Since all the phases are in equilibrium with each other, it is difficult to define the exact concentration of material to which an organism is exposed.

These factors are relevant to both

- testing of surface-active substances, and
- the use of surfactants as vehicles for a test substance.

3.1.3 Colloidal Dispersions

When particles of a substance have a diameter of about 1 μm or lower, stable dispersions can form, which, according to whether the substance is a liquid or solid will be termed emulsions or suspensions (Bahadir *et al*, 1995). If the size of these particles is lower than 0.1 μm , the heterogeneous character of the suspension or emulsion may not be visible, particularly at low concentrations. Its stability with respect to sedimentation or aggregation increases as the size of the particles decreases.

Some substances, for example oil-based products, can form stable dispersions when stirred in water with excess substance present. The exact nature of the dispersion, and its properties, may depend upon the method used to form the dispersion, and the amount of substance used.

3.2 ADSORPTION

As a result of high adsorptive tendency, substances may be lost from solution due to adsorption onto the surfaces of the test vessel, to the test organisms or to particulate matter. The potential for adsorption to particulate matter can be estimated from the organic carbon/water partition coefficient (K_{OC}). Adsorption onto glass surfaces, which may occur with substances of very low solubility, can only be determined experimentally.

3.3 HIGH VOLATILITY

Aquatic toxicity testing is normally conducted in open systems. To maintain the oxygen concentration needed for the survival of the test organisms, air exchange with the atmosphere is often required. Under such circumstances, volatile substances have the potential to be lost to the atmosphere by evaporation. The vapour pressure of the substance and its Henry's constant are important parameters which describe that potential, and whether the test system design needs to account for volatility of the substance.

3.3.1 Henry's Constant

The Henry's constant of a substance is a measure of its equilibrium between an ideal solution phase and the vapour phase. In practical terms, a Henry's constant of $> 0.1 \text{ Pa m}^3 \text{ mol}^{-1}$ will give rise to a loss of substance at rates that are important relative to the length of typical short-term ecotoxicity tests although it is dependent on the test design (Mackay, 1992). With respect to aquatic toxicity testing, the Henry's constant is the best measure of the potential for loss of the dissolved substance from the water by volatilisation.

3.3.2 Vapour Pressure

The vapour pressure of a substance is a measure of its equilibrium between a condensed phase and the vapour phase. Vapour pressure may also be relevant if undissolved material is present, for example as a surface film in contact with the gas phase: significant transfer to the vapour phase would cause a change in the amount of undissolved substance present, and, for complex substances, a change in the composition of the medium. The maximum amount of substance lost to a known volume of vapour space can be calculated by assuming ideal gas behaviour and the volume of a mole of substance at the temperature and pressure of the system.

3.4 INSTABILITY

Instability of the substance caused by oxidation in water, hydrolysis, photolysis and/or biodegradation can reduce the concentration of the parent substance in the test medium and increase the level of breakdown products. These products can be more or less toxic than the parent substance; usually they are more water soluble than the parent.

3.4.1 Hydrolytic and Oxidative Stability

Half-lives for substances which undergo hydrolysis vary from less than seconds to months or years. There are many functional groups which may undergo significant hydrolysis in aquatic test media; typical examples are epoxy groups, activated esters, isocyanates, carbamates and acid chlorides.

Oxidation in water has not been described in the literature as extensively as hydrolysis. Functional groups susceptible to oxidation include phosphines, alkylated phenols, aromatic diols, metals in low oxidation states and aldehydes.

3.4.2 Photodegradation

For a substance which absorbs light in the range 290 to 600 nm, there is a potential of photodegradation: light of the shorter wavelengths of the solar spectrum has sufficient energy to break chemical bonds (Leifer, 1988; Schwarzenbach, 1993). Most indoor lighting sources are weak in the UV spectrum, so in laboratory testing the problem should be minimal. However, where a chemical displays pronounced light absorption, precautionary measures may be necessary.

3.4.3 Biodegradation

Ecotoxicological tests are not usually conducted in sterile environments and therefore the potential exists for biodegradation of the substance. Losses of the test substance due to biodegradation tend to increase with time (after a lag time which cannot be predicted) and to result in changes in the test medium, e.g. oxygen depletion, pH fluctuation.

3.5 COMPLEX SUBSTANCES

Complex substances may pose a problem particularly where one or more of the components is sparingly water soluble. Where the solubility varies, the actual composition of the test medium will depend on the method of preparation, e.g. a Water Accommodated Fraction (WAF) (see Appendix C). The rate of attainment of a steady solution composition may be slow requiring several days for media preparation. Finally, for mixtures containing multiple components, it may be difficult to express the concentration of the mixture in solution as most analytical methods will only identify specific compounds. Accurate presentation of the concentration of the mixture is difficult. For tests with pure compounds, the correct measure of exposure is the concentration in solution. Alternatively, if a dispersion of the compound is tested the concept of the loading-rate may be more appropriate (CONCAWE, 1993). The loading rate is the quantity of test compound per unit volume of water used in the preparation of the test medium. For poorly water-soluble substances the loading rate will usually not be the same as the concentration of any individual compound in solution.

4. USE OF AUXILIARY AGENTS IN TOXICITY TESTS

Two types of auxiliary agents can be distinguished, namely water-miscible solvents and dispersants or emulsifiers (e.g. surfactants). Auxiliary agents are commonly used in routine testing for a number of purposes:

- to facilitate handling of concentrated solutions of test substances that are not readily water soluble prior to dosing;
- to prevent hydrolysis of the test substance in stock solutions;
- to support stable dispersions;
- to provide "exposure" to test substances above their water solubility while minimising or eliminating visible test material particles;
- reduce interface tension to improve wetting.

The OECD and EEC test guidelines recommend a limit of 100 mg/l of auxiliary agent in the exposure test medium and that the auxiliary agent should not be toxic in the concentration range used. The toxicities of some solvents frequently used in aquatic toxicity tests are shown in Tables 2 and 3.

Based on these data, the use of solvents up to 100 mg/l in acute toxicity tests should not have a toxic effect. However, the use of solvents which are readily biodegradable, such as acetone, ethanol and methanol may result in oxygen depletion of the test medium. This problem can be overcome by elimination of the solvent prior to the test or can be reduced by using solvents such as dimethylformamide (DMF) or triethylene glycol, which have a low degradability and a high ability to dissolve many organic substances.

4.1 EFFECTS ON SOLUBILITY IN THE TEST MEDIUM

4.1.1 Solvents

Although the OECD/EEC test guidelines now provide guidance on the use of solvents at concentrations up to 100 mg/l, published literature reveals that in the past much higher concentrations have been employed. Solvents at low concentrations do not significantly influence the water solubility in accordance with basic physical/chemical principles. Exceptions have been reported which may, however, be explained by a higher velocity of solution or by interactions between the solvent and the test substance. For example, Herzel and Murty (1984) showed that acetone at concentrations in the range 10 to 500 µl/l (7.9-400 mg/l) did not increase the water solubility of dieldrin and nitrofen, but increased the apparent water solubility of captan significantly.

Table 2: Toxicity of Some Typical Solvents in Acute Tests

Auxiliary agent	Species	Concentration mg/l	Endpoint (Test Duration)	Reference
Acetone	<i>Oncorhynchus mykiss</i>	5500	LC50 (96h)	Johnson and Finley, 1980
	Brook Trout	6070	LC50 (96h)	US-EPA, 1982
	Fathead minnow	9100	LC50 (96h)	US-EPA, 1982
	<i>Daphnia magna</i>	39000	EC50 (48h)	Leblanc and Surprenant, 1983
	<i>Daphnia magna</i>	1230	EC50 (18h)	Bowman <i>et al</i> , 1981
	<i>Daphnia magna</i>	12700	EC50 (96h)	Adema, 1980
	<i>Chlorella pyrenoidosa</i>	30200	EC50 (not specified)	Stratton and Smith, 1988
Dimethyl sulphoxide DMSO	<i>Oncorhynchus mykiss</i>	35000	LC50 (96h)	Johnson and Finley, 1980
	<i>Daphnia magna</i>	27500	EC50 (18h)	Bowman <i>et al</i> , 1981
	<i>Daphnia magna</i>	17600	EC50 (96h)	Adema, 1980
	<i>Artemia salina</i>	6500	EC50 (72h)	Barahona-Gomariz <i>et al</i> , 1994
	<i>Chlorella pyrenoidosa</i>	20100	EC50 (not specified)	Stratton and Smith, 1988
Dimethyl formamide DMF	<i>Oncorhynchus mykiss</i>	9800	LC50 (96h)	Poirier <i>et al</i> , 1986
	Brook Trout	8366	LC50 (96h)	US-EPA, 1982
	Fathead minnow	10410	LC50 (96h)	US-EPA, 1982
	<i>Daphnia magna</i>	13000	EC50 (48h)	Leblanc and Surprenant, 1983
	<i>Daphnia magna</i>	16200	EC50 (96h)	Adema, 1980
	<i>Chlorella pyrenoidosa</i>	9400	EC50 (not specified)	Stratton and Smith, 1988
	Triethylene glycol	Brook Trout	73500	LC50 (96h)
Fathead minnow		92500	LC50 (96h)	US-EPA, 1982
<i>Daphnia magna</i>		35000	EC50 (48h)	Leblanc and Surprenant, 1983
<i>Microcystis aeruginosa</i>		3600	Threshold value ^a	Bringmann and Kühn, 1976
Methanol		<i>Oncorhynchus mykiss</i>	19000	LC50 (96h)
	<i>Daphnia magna</i>	>10000	EC50 (24h)	Bringmann and Kühn, 1982
	<i>Daphnia magna</i>	19600	EC50 (18h)	Bowman <i>et al</i> , 1981
	<i>Daphnia magna</i>	15900	EC50 (96h)	Adema, 1980
	<i>Artemia salina</i>	9000	EC50 (72h)	Barahona-Gomariz <i>et al</i> , 1994
	<i>Chlorella pyrenoidosa</i>	36000	EC50 (not specified)	Stratton and Smith, 1988
	Ethanol	<i>Oncorhynchus mykiss</i>	13000	LC50 (96h)
<i>Daphnia magna</i>		12000	EC50 (48h)	Takahashi <i>et al</i> , 1987
<i>Daphnia magna</i>		12100	EC50 (18h)	Bowman <i>et al</i> , 1981
<i>Daphnia magna</i>		14200	EC50 (48h)	Adema, 1980
<i>Ceriodaphnia dubia</i>		5-6000	EC50 (48h)	Takahashi <i>et al</i> , 1987
<i>Artemia salina</i>		7000	EC50 (72h)	Barahona-Gomariz <i>et al</i> , 1994
<i>Chlorella pyrenoidosa</i>		11800	EC50 (not specified)	Stratton and Smith, 1988

^a concentration at which inhibition of cell multiplication begins

Table 3: Toxicity of Some Typical Solvents in Chronic Tests

Auxiliary agent	Species	Concentration mg/l	Endpoint (Test Duration)	Reference
-----------------	---------	--------------------	--------------------------	-----------

Acetone	<i>Daphnia magna</i>	1400-2800	MATC (28d)	Leblanc and Surprenant, 1983
Dimethyl formamide	<i>Daphnia magna</i>	1200-2500	MATC (28d)	Leblanc and Surprenant, 1983
DMF				
Triethylene glycol	<i>Daphnia magna</i>	5500-11000	MATC (28d)	Leblanc and Surprenant, 1983

MATC: Maximum Acceptable Toxicant Concentration

At high solvent addition levels (mole fraction = 0.05 - 0.1), increases in solubility have been observed for a variety of organic compounds (Nyssen *et al*, 1987). This has been confirmed by Li and Andren (1994) for polychlorinated biphenyls with volume fractions > 0.05 of various alcohols.

4.1.2 Surfactants

Surfactants are substances which usually are soluble in water and in water-immiscible liquids. At low concentrations they accumulate at interfaces and surfaces thus reducing interface and surface tensions. When the interfaces and surfaces are fully occupied and the concentration of the surfactants in water exceeds solubility, several of their molecules form aggregates called micelles with the hydrophobic moieties of the molecules directed to the centre. The concentration at which micelle formation begins is called Critical Micelle Concentration (CMC).

Limited data are available on the effects of surfactants when used as auxiliary agents, although they are widely used to prepare stable dispersions of test substances and to overcome surface tension effects in tests with organisms such as *Daphnia* (entrapment of *Daphnia* in surface film). Surfactants can interact with organic substances both in the micellar and monomeric form to increase the water solubility (Edwards *et al*, 1991; Edwards *et al*, 1992). This effect is well known above the CMC and is considered to be due to partitioning into the micelle. At surfactant concentrations below the CMC, the apparent solubility of phenanthrene and pyrene were increased by a factor of approximately 3 over the pure water solubility (Edwards *et al*, 1992). Such effects are believed to be due to interactions between the substance and the hydrophobic region of the surfactant giving rise to a surfactant-organic substance complex. The hydrophilic region of the surfactant interacts with water molecules, thus reducing the forces driving the substance out of solution. Kile and Chiou (1989) observed that the surfactants Triton X-100 and X-114 (octylphenol ethoxylates) and Brij-35 (polyoxyethylene lauryl ether) significantly increased the water solubility of DDT, when used at concentrations of 200 mg/l and above. A less pronounced effect was observed on the water solubility of trichlorobenzene. Jafvert *et al* (1994) have reported the solubilisation of hexachlorobenzene (HCB) using non-ionic surfactants. The solubility of HCB in distilled water was approximately 5 µg/l and this increased linearly with surfactant concentrations

above the CMC. At a concentration of 0.5 g/l of Tween 85 (alkyl sorbitan ethoxylate) the solubility of HCB was 600 µg/l.

4.2 EFFECTS ON TOXICITY OF TEST SUBSTANCE

Solvents

There are few reports on the comparative effect of solvents on toxicity measurements. Calleja and Persoone (1993) showed that the presence of solvents (dimethylsulfoxide (DMSO), ethanol, methanol and acetone) could modify the acute toxicity of some poorly soluble organic compounds to aquatic invertebrates. The most striking effects were observed with malathion, which was approximately 10 orders of magnitude less toxic in the presence of DMSO and acetone in concentrations of 1% v/v (11,000 mg/l DMSO and 7,900 mg/l acetone) than when tested using water alone. Although the authors did not report the solubility of the organic compounds used in the test, calculation of solubility from K_{ow} values suggest that all compounds were tested below their solubility limits. On the other hand, DMSO and ethanol increased the toxicity of diazepam to daphnids and rotifers, but reduced its toxicity to *Artemia*. The authors concluded, since the mechanisms for synergism and antagonism for mixtures are poorly understood, the use of solvents should be avoided, if possible. However, these conclusions were based on solvent concentrations in excess of those permitted by the test guidelines (max. 100 mg/l). Therefore, these observations and conclusions are not relevant to aquatic toxicity tests performed according to standard guidelines.

Berglind and Dave (1981) reported that the addition of acetone to the test medium at a concentration of 0.5 ml/l (395 mg/l) did not influence the toxicity of HDEHP (hydroxy diethyl hydrophosphonic acid) or Aliquat 336, a long chain quaternary ammonium chloride, compared to their toxicity alone. However, preparation of test solutions of Aliquat 336 in acetone reduced the acute toxicity relative to stock solutions prepared in water with sonication. Shubat *et al* (1982) also observed that the use of dimethyl formamide (DMF) (0.5 mg/l) as a solvent did not alter the toxicity of tetrachloroethylene to rainbow trout.

Surfactants

There are even fewer reports on the comparative effect of surfactants on toxicity measurements. The physical form of the substance may influence distribution/solubility and hence, have an unknown and unpredictable effect on uptake (Ariens, 1971). Poremba (1993), in studies to assess the effect of dosing agents on the toxicity of hydrocarbon mixtures, observed that when surfactants were used to disperse the hydrocarbons, the toxic effect level varied according to the type and quantity of surfactant used.

4.3 CONCLUSIONS

Two different classes of auxiliary agents used in standard toxicity testing were identified, i.e. organic solvents and surfactants.

Low concentrations of solvents do not significantly influence the water solubility of a substance. Apparent exceptions can be explained according to known mechanisms. Surfactants can interact with organic substances to increase the apparent water solubility at low concentrations.

The acute EC_{50} of the common solvents used in standard testing has been demonstrated to be much higher than 100 mg/l. Based on the limited information available, it can be assumed that chronic effects only occur at higher concentrations. However, it has been shown that the presence of solvents could modify the toxic effects of some sparingly soluble substances though these examples typically use solvent concentrations higher than 100 mg/l. A limitation for the use of solvents like acetone, ethanol or methanol is their ready biodegradability and hence, oxygen depletion in the test solution. Elimination of such solvents prior to the start of the test by application of adequate methods may reduce such problems. Alternative solvents are dimethylformamide (DMF) or triethylene glycol, which have a low volatility and a high ability to dissolve many organic substances, while reducing the problem of oxygen depletion.

Generally, the use of solvents should be restricted to those cases where a stock solution has to be prepared with a hydrolytically unstable substance, or the test substance could not be dispersed by other means, e.g. for highly viscous substances.

The use of surfactants as dispersing agents should be avoided, due to their profound effect on the physical form of the test substance in the test medium.

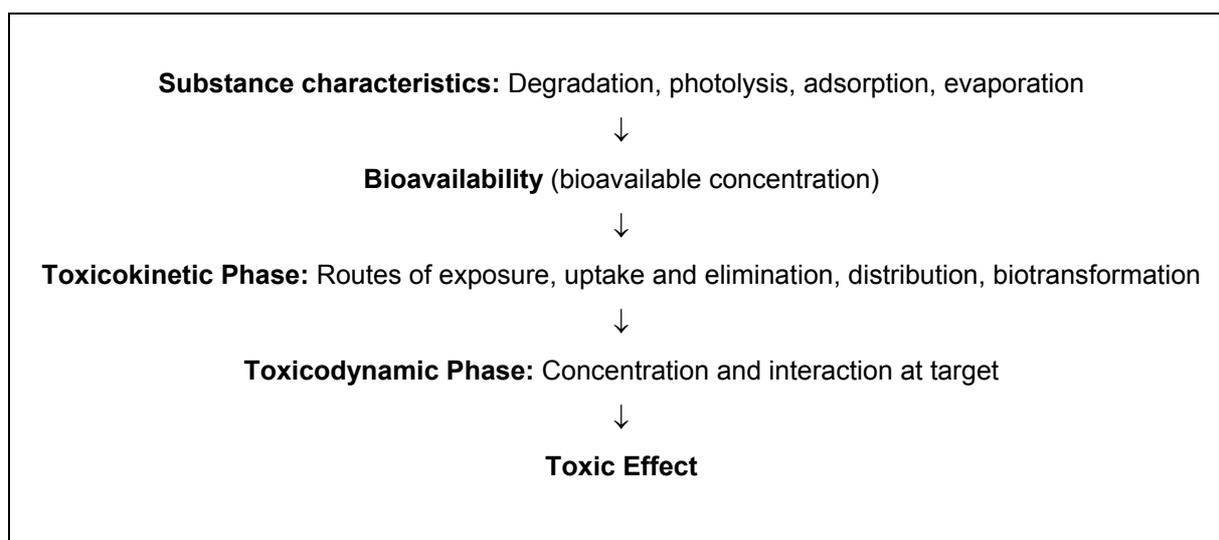
5. REVIEW OF UPTAKE MECHANISMS AND THEIR SIGNIFICANCE FOR AQUATIC TOXICITY

The purpose of this section is to review the uptake mechanisms of substances by organisms in the aquatic environment and the significance for toxicity testing in the laboratory.

5.1 AQUATIC TOXICITY

A toxic effect is defined as an adverse biological effect arising from the interaction of a substance with a specific target or targets (i.e. organ / biochemical mechanism / physiological process) in the organism. The magnitude of this toxicological response is a function of the amount or concentration of the toxicant reaching the target site and time. In aquatic toxicity testing it is generally not feasible to measure the concentration at the target site and therefore the observed toxicity is related to the concentration in the test medium. This rationale assumes that the internal body concentration is proportional to the external concentration in water. However, there are many factors that may affect such a relationship and much of the inter-species differences in the toxicity of a substance are thought to be due to differences in the physiological and biochemical processes that govern the rates of uptake, metabolism, distribution and excretion. The type of test system and abiotic variables, such as pH, water hardness, temperature and the presence of organic matter may also contribute to differences in observed toxic effects (see Fig. 1).

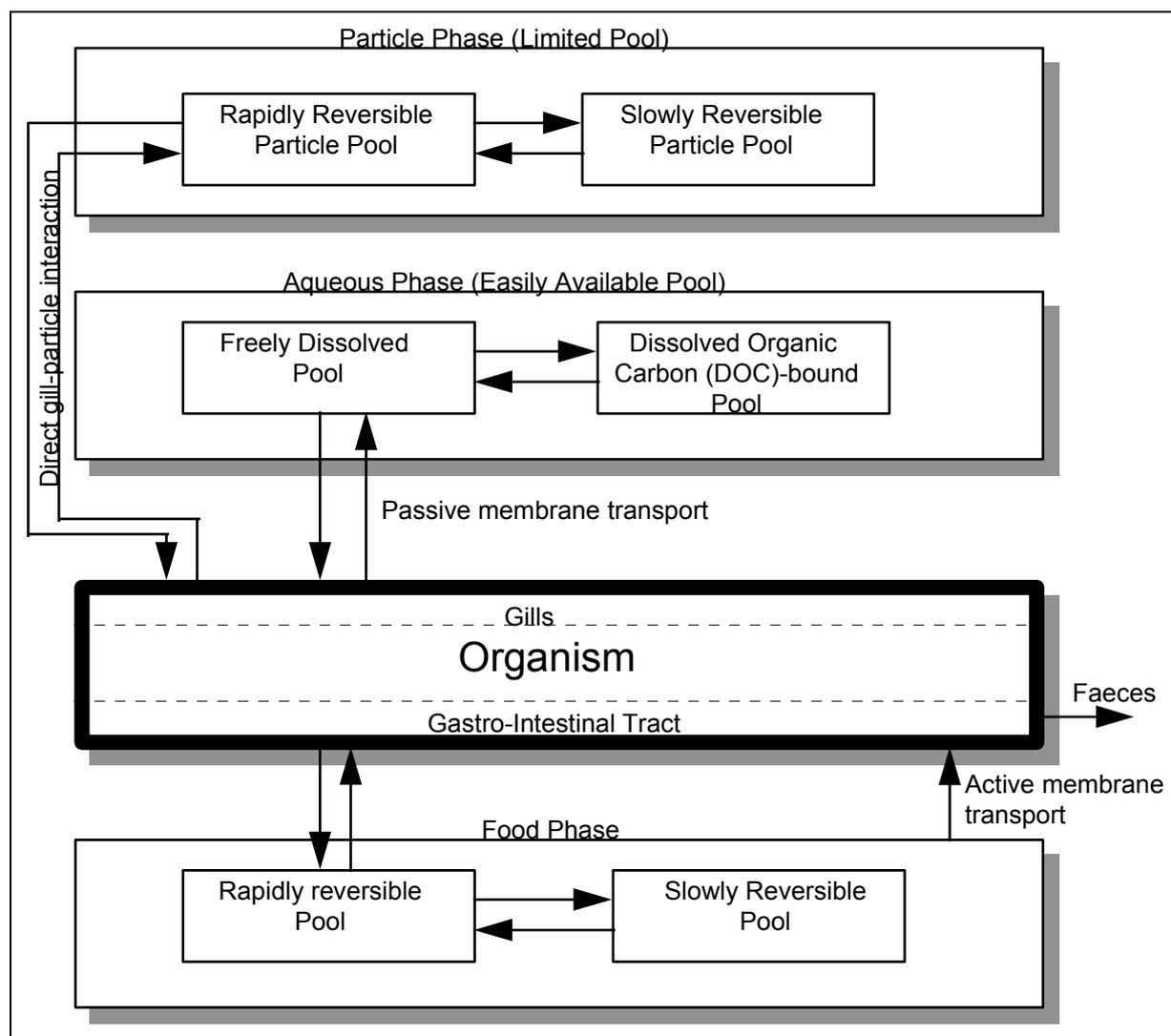
Fig. 1: Factors Influencing the Observed Toxic Effect of a Substance



5.2 UPTAKE ROUTES

Substances to which aquatic organisms are exposed may be present in the aquatic environment in three different phases (see Fig. 2):

Fig. 2: Concentrated Models of the Different Pools of Toxicants, their Relations and the Uptake Mechanism



- the aqueous phase in which the test substance is present as a true solution ("freely dissolved pool") or associated with dissolved organic carbon (DOC) ("DOC-bound pool");
- the particle phase which consists of undissolved particles of the test substance. From this phase the test substance can enter the aqueous phase by desorption from the surface ("rapidly reversible pool") and from the particle phase ("slowly reversible pool");

- the food phase, in which the test substance is associated with the food. The dissolution from the food may be comparable to that from the particle phase and can therefore also be divided into a "rapidly reversible pool" and a "slowly reversible pool".

Uptake of the test substance may take place from all three phases by different mechanisms:

- uptake directly from the aqueous phase via absorption through membranes (e.g. the respiratory surface, gills) or the skin;
- uptake from the particle phase at the respiratory surface (e.g. via gills/particle interaction);
- ingestion from the food phase.

The relative significance of these uptake pathways varies with the organism and the attributes and mode of presentation of the substance being investigated. For substances with $\log K_{ow} < 4.5-5$, uptake in fish is predominantly through the gill and direct absorption across the skin (ECETOC, 1996). The relative proportion of uptake via the gill and skin depends upon the biology of the organism (size, presence of scales, blood flow to the skin, etc.). According to Lien and McKim (1993), diet (ingestion) can be the major route of uptake for substances with $\log K_{ow} > 5$. Various uptake mechanisms exist, such as passive and facilitated diffusion, active transport, pinocytosis, and phagocytosis (reviewed in Appendix D). However, there is little evidence that mechanisms other than passive diffusion are important in the uptake of substances (ECETOC, 1996).

Diffusion is limited by the molecular size of the substance. Above a certain molecular size, which experimental studies show to be equivalent to a cross sectional area of about 1 nm^2 (Opperhuizen, 1986; Anliker *et al*, 1988), or a molecular weight of about 700 (EEC, 1993b), steric hindrance restricts the diffusional transport of a chemical across a biological membrane, such as gut or gills. The data of Opperhuizen (1986) and Anliker *et al* (1988) further support the minor role of pinocytosis and phagocytosis on particulate uptake.

5.3 FACTORS INFLUENCING UPTAKE

The duration of exposure and the bioavailability of a substance are two important parameters which influence uptake.

5.3.1 Duration of Exposure

Acute toxicity testing is normally conducted over a short time period (typically 48 or 96 hours). Material from the "freely dissolved" pool (see Figure 2) may be absorbed and will ideally attain an equilibrium between internal and external concentration. For sparingly soluble substances with undissolved material

present, however, the exposure time may be too short to permit an equilibrium to be established between the "freely dissolved", the "reversible" (limited) pool and the organism within the period of the test (Figure 2). This is supported by experimental data from Hawker and Connell (1985), who showed that 96 h (acute) and even, in some cases, 14 d (subchronic) tests were too short to establish an equilibrium. Hermens *et al* (1984) identified that for substances with a high K_{ow} - which is normally associated with low water solubility - a "cut-off" point exists, above which the 96 hour LC_{50} can no longer be determined because a toxic threshold is not reached within 96 hours in the organism. For some substances a toxic threshold may be reached with longer exposure while for others it appears probable that, even with infinite exposure, no threshold will ever be reached (see also: ECETOC, 1996).

For chronic testing, an equilibrium will be maintained between the aqueous and particle phase. As the dissolved substance in the aqueous phase is absorbed by the organism, the aqueous concentration is maintained by dissolution from the undissolved substance from the particle phase. As a result of increased test duration, the K_{ow} driven "cut-off" may increase enabling a NOEC value to be determined. In this case, the particle phase is only serving as a reservoir to replenish the aqueous phase. Low dissolution rates, however, could result in a decrease of exposure concentrations in the aqueous phase. In these cases, a toxicity threshold may not be achieved during the period of the test although equilibrium is established between the aqueous phase and the organism. Flow-through testing at the solubility limit achieves the same purpose by maintaining a constant concentration of the substance in the aqueous phase whereas in the static test, the limited rate of dissolution of particles may result in a decreased exposure concentration (decreased concentration in aqueous phase).

Thus, the time to reach equilibrium between the test organism and the test medium is dependent on the test conditions and the test organisms, but also on the physico-chemical properties of the test substance, e.g. the K_{ow} , rates of uptake and depuration. The duration of a toxicity test may therefore need to be adjusted to the properties of the test substance (for guidance see ECETOC, 1996). However, test guidelines do not allow this flexibility.

5.3.2 Bioavailability

Bioavailability refers to that proportion of the total environmental loading of a substance (present in the water, suspended solids or food), which is available for uptake by an organism (Spacie and Hamelink, 1985). A number of factors will modify the bioavailability of substances in laboratory test systems and in the natural aquatic environment. To be bioavailable for transfer across biological membranes, a chemical needs to be present in water in the dissolved fraction, but uptake is limited by the molecular size of the substance (see 5.2).

A number of studies (McCarthy and Jimenez, 1985; Servos *et al*, 1989; Black and McCarthy, 1988; Versteeg and Shorter, 1992) have shown that the presence of dissolved organic material (DOM) or particulate matter, reduces the availability of chemicals. Sediment has also been shown to have a similar effect (Knezovich and Harrison, 1988; Schrap and Opperhuizen, 1990). The extent to which this phenomenon occurs cannot be quantified although it appears that the physico-chemical properties of the test substance and the nature of DOM or particulate matter dictate the extent of sorption (Versteeg and Shorter, 1992).

5.4 CONCLUSIONS

In expressing toxic effects, aquatic organisms respond to the internal or tissue concentration of the substance of interest. Substances are taken up primarily via the respiratory surface, but also through absorption across the skin and gastrointestinal tract following ingestion. For any given species and age class, the relative significance of each uptake pathway will be dependent on the chemistry of the substance (e.g., less soluble and more hydrophobic molecules accumulating to a greater degree from the food chain). The tissue concentration achieved will be proportional to the exposure duration and the bioavailable concentration. As there is limited evidence to suggest that particulate uptake occurs to any significant extent, the rate constant for uptake of a substance can be taken as directly proportional to the concentration of soluble substance. Therefore, there is no advantage in testing above the water solubility limit, except for gaining information on the presence of toxic soluble impurities.

6. POSSIBLE EXPLANATIONS FOR ADVERSE EFFECTS ABOVE THE LIMIT OF WATER SOLUBILITY

For some substances, test results may exhibit a concentration-toxicity response relationship under conditions, where the water solubility of the substance has been exceeded. This section considers some of the most probable reasons why adverse effects above the water solubility may occur.

6.1 EFFECTS OF MINOR COMPONENTS

Ideally, a test substance will have been characterised (to the extent necessary) and its composition described. Solubility (and other physical and chemical properties) are usually established for the major component(s) only. Minor components or impurities present may have different solubility characteristics. When dosed into a test above the solubility limit of the primary component, the concentration of impurities in solution may continue to increase as further test substance is added, until the solubility limit of the impurities is reached. These soluble components (impurities) may express toxicity, which is valid for the substance tested but is often erroneously attributed to the named major component of the substance and not to the impurities.

An example of the effects of minor components is DTDMAC (ditallow dimethyl ammonium chloride). It is a practically insoluble substance (solubility estimated as $<10^{-15}$ mol/l) which forms liquid crystals in water (Versteeg *et al*, 1992; ECETOC, 1993b). A number of toxicity studies with commercial DTDMAC have demonstrated good dose response relationships for a variety of aquatic species. Commercial DTDMAC typically contains 3 to 8% of the water soluble monotallow trimethyl ammonium chloride (MTTMAC). This substance has an appreciable toxicity to aquatic organisms and much or all of the toxicity ascribed to commercial DTDMAC appears to be due to MTTMAC (Versteeg *et al*, 1992).

6.2 EFFECTS OF THE INSTABILITY OF THE TEST SUBSTANCE

When an unstable (reactive) substance is present in water, degradation can occur giving rise to a supply of dissolved reaction products that may be toxic. In most cases, the products of degradation (of any type) are more water soluble than the parent.

The concentration of reaction products in solution will depend upon:

- the quantity of excess substance present initially;
- the physical form of the excess substance and the overall rate of reaction;
- the time elapsed since the substance was mixed with water;

- the loss rate of the reaction products (e.g. volatilisation).

For such substances, dose and time dependent toxicity could therefore be expected even at levels far exceeding their solubility.

6.3 PHYSICAL EFFECTS

Physical rather than toxic effects caused by undissolved substances can affect filter feeding organisms and organisms with gills or other respiratory surfaces which are exposed to the external water environment. Since filter feeding and respiration usually requires relatively large volumes of water to be passed over the surface of the organ, there is a high potential for aggregates physically to impair their function.

Entrapment of *Daphnia* in surface films of undissolved test substance is a commonly observed phenomenon in ecotoxicological tests. Entrapment results in fouling of the respiratory and feeding organs and in restricted mobility. In fish tests, adsorption of aggregates or surface films onto gill surfaces inhibits oxygen uptake and ionoregulation. These effects can result in immobility or death even though the substance itself may not be toxic at the concentrations causing these indirect effects.

A further physical effect can occur with algae; they can aggregate on particles of undissolved test substance. The result can be reduced algal growth, possibly due to reduced access of nutrients. This problem may be due to the negative surface charge of the cell membrane interacting with cationic particulates.

6.4 EFFECTS OF LIGHT ATTENUATION

Insoluble substances are capable of attenuating light penetration into the test medium, by light absorption and reflection. When the test substance forms a homogeneous dispersion in water, attenuation of light is likely to be proportional to the amount of substance added. The inhibition of algal growth due to light attenuation can result in reduced algal population growth in relation to the amount of substance added to the test medium. This is not a true toxicity effect.

6.5 EFFECTS OF PARTICULATE UPTAKE

Where the water solubility limit of the test substance in the test medium is exceeded, particles of undissolved substance will be present. These particles can be dispersed in solution and can be an appropriate size for ingestion. It is possible that direct ingestion of particles of the substance by the organism could occur. The significance of this mode of uptake is uncertain.

The only study which documents a toxicological effect at exposure levels above the water solubility is a recent study by Potter *et al* (1994). They looked at the effect of benzo[a]pyrene (BaP) on the formation of DNA adducts in the trout liver. Two groups of fish were used; the first were exposed to between 4 and 16 µg/l of an aqueous dispersion of BaP for 1-2 days (BaP has a water solubility of 0.4 µg/l), followed by lower concentrations for the remainder of the 30 day test period. Average concentrations between days 15 and 30 were 0.5 µg/l. No increase in DNA adduct formation was detected. In the second group, exposure of 0.5 µg/l for 15 days followed by a peak exposure of 60 µg/l (which then dropped to 2 µg/l by day 30) resulted in DNA adduct formation in the liver. The authors attributed this to the uptake of BaP in particulate form, at concentrations considerably exceeding water solubility. However, no measure of total body burden of BaP was made and the typical exposure pattern, combined with the absence of information on DNA adduct turnover rate in trout suggests that this conclusion should be regarded with caution.

By using radiolabelled samples of the test substances, Marshall and Van Egmond (1995) were able to assess the critical body residues (CBR)³ in the fish when exposed to a dispersion of phenanthrene, musk xylene, hexadecanol and lauric acid at total concentrations that exceeded the water solubility limit. They found that the CBRs were strongly related to the estimated soluble fraction, suggesting that particulate test substance present in the medium did not interact with the test organisms. They estimated the soluble fraction by separation of excess test material either by centrifugation at 40,000 g for 30 min or by double filtration through a 0.22 µm filter.

Two exposure scenarios that require more detailed investigation are the possible interaction of particulates with filter feeders, such as daphnids, and possible interactions between organisms and droplets or dispersions of liquid substances.

³ The concentration of a substance in an organism at the time of death or any other biological end-point

6.6 CONCLUSIONS

For some substances, test results may show a concentration-toxicity response relationship above the water solubility limit. The most probable reasons for such relationships are either artefacts of the test procedure (like physical effects by undissolved material or direct ingestion of particles of the substance by the test organism), which are not due to the intrinsic toxicity of the test substance, or the fact that effects were incorrectly attributed to the named substance itself, while they were caused by impurities or transformation products which often display better water solubility. The results obtained under such conditions should be interpreted with particular care, even if the likely mechanism is known. It should be kept in mind, however, that in the EU the properties of the total substance as put on the market, including impurities, are important for, e.g., classification and labelling as well as risk assessment.

7. STRATEGY FOR TESTING AND INTERPRETATION OF TEST RESULTS

This section presents an approach to testing all substances on the basis of the preceding information.

Detailed guidance on how to perform aquatic toxicity tests on difficult substances has been given in a number of publications by other organisations; the Task Force endorses many, but not all of the recommendations made in these reports. The document prepared for the Chemical Notification Unit of the UK Department of the Environment (Whitehouse and Mallet, 1993), provides comprehensive coverage for all the classes of difficult-to-test substances. This document is being revised by Marshall and van Egmond (unpublished) based on discussions with relevant experts and on the recommendations produced by the UK Ecotoxicity Shadow Group (Stephenson, 1992). Both the original document of Whitehouse and Mallet (1993) and its revision (when completed) may be obtained from the Chemical Notification Unit of the UK Department of the Environment. The main points of these two documents are summarised in this report but for a comprehensive account the reader should obtain the original document(s). Specific guidance on testing complex hydrocarbon mixtures is given in a report prepared by and available from the Oil Companies' European Organisation for Environmental and Public Health Protection (CONCAWE, 1993). This approach is now included in the EU Technical Guidance Document for risk assessment of existing substances (EEC, 1994). A summary of the key points of this document is provided in Appendix C. An International Standards Organisation draft document (ISO, 1994) describes the procedures for determining the toxicity of difficult-to-test substances to algae. It generally suggests the same procedures as the document by Whitehouse and Mallet (1993).

The Task Force also recognises the recommendations prepared by the UK Ecotoxicity Shadow Group (Stephenson, 1992). Where relevant, these have been included in this section.

Specific recommendations or comments of the Task Force are outlined below and a scheme to aid test design is presented.

7.1 PERFORMANCE OF TEST

Information on the composition and identity of the test substance, as well as appropriate physico-chemical data should be available prior to performing any test, so that the likely behaviour of the substance(s) may be predicted and the appropriate experimental design employed. Such requirements apply to all substances, but are particularly relevant when testing and interpreting the data on difficult-to-test substances.

In reviewing the physico-chemical data required for substances, the Task Force has highlighted some of the deficiencies in the standard methods used to obtain these data (see Appendix A).

7.1.1 Stability assessment

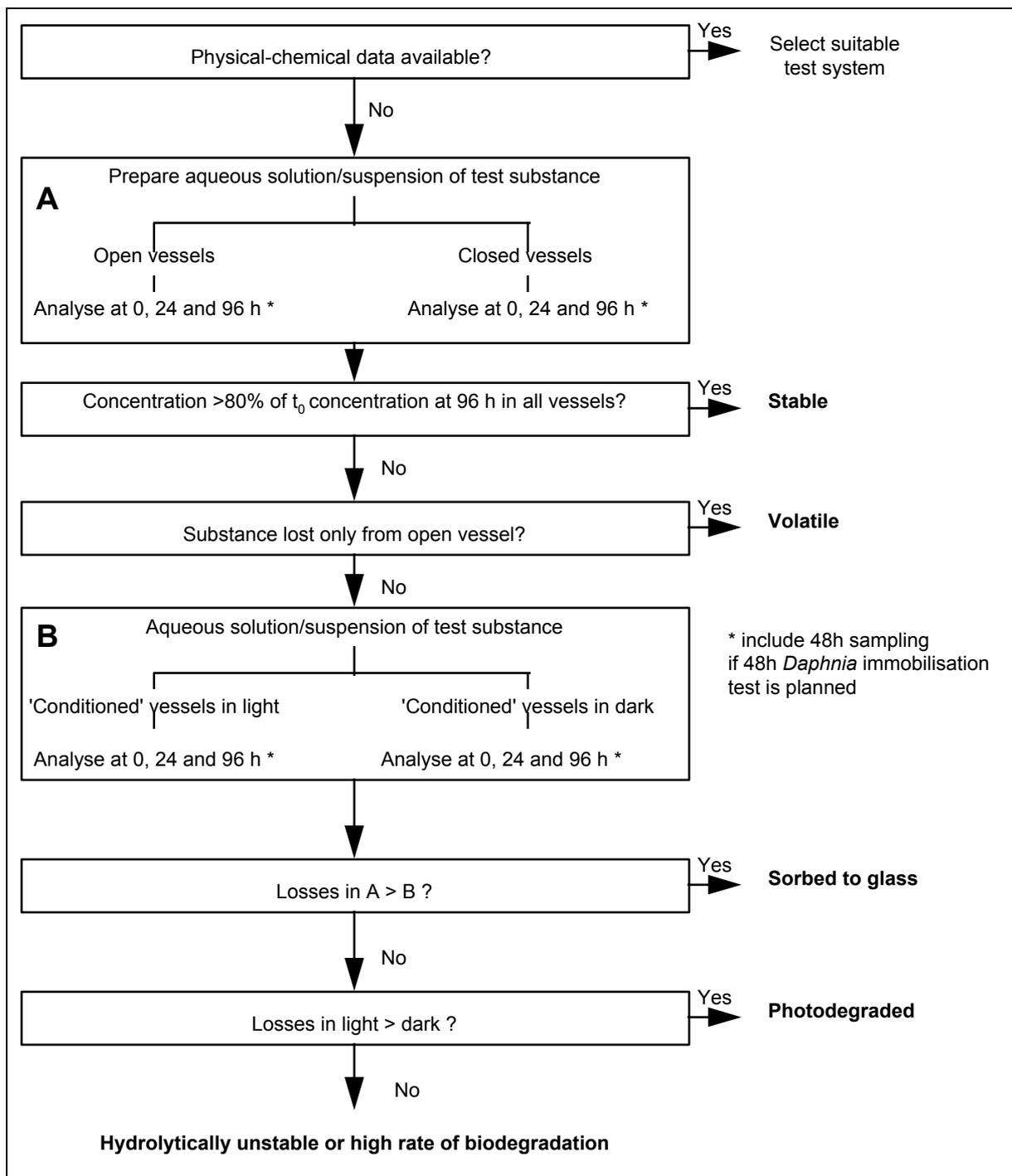
A critical feature of any test is the maintenance of the exposure concentration throughout the period of the test. Therefore, prior to performing any tests it is essential that an assessment of the likely exposure conditions and the test concentrations is performed. If the physico-chemical data for the base set is available, then it is usually possible to design the toxicity test without recourse to a pre-stability test as described by Whitehouse and Mallet (1993). However, if the relevant data are not available, then it is advisable to determine the stability and solubility of the test substance under the conditions of the proposed toxicity test and to design the exposure scenario accordingly. A flow chart (from Whitehouse and Mallet, 1993) summarising the options and the likely routes of loss is shown in Figure 3. Technical measures to minimise losses are discussed in Section 7.1.3.

7.1.2 Media preparation and use of auxiliary agents

General Principles

The Task Force recommends that the preferred options for preparing test solutions are physical methods, such as stirring or sonication. The use of ball mills and/or vacuum may be helpful for substances which are difficult to wet. Column generator systems are also useful, but have limited applications and the composition of the final medium should be assessed. For example, in the case of hydrolytically unstable substances or substances containing components with very different water solubilities, the saturated medium may be very different in composition compared to the original test substance. Details of the preparation of media are described in Whitehouse and Mallet (1993).

Fig. 3: Flow Chart Summarising the Options and Likely Routes of Loss according to Whitehouse and Mallet (1993)



Solvents

The Task Force recognises that solvents may be essential in handling some substances, for example for preparing stock solutions of hydrolytically unstable or highly viscous substances, and to aid dispersion and subsequent dissolution in test media. A limitation in the use of solvents, such as acetone, ethanol and methanol, is their ease of biodegradability which may result in oxygen depletion of the test medium. Elimination of such solvents prior to the start of the test by application of adequate methods may reduce such problems. Alternative solvents are dimethylformamide or triethylene glycol, which are not as easily biodegraded. For substances containing a number of components, solvents should be selected in which the components are fully soluble.

Dispersants

The use of dispersants, which are usually surfactants, should be avoided. Dispersants even if non-toxic may have a pronounced effect on the physical form of the test substances in the test medium and may thereby directly or indirectly influence their bioavailability. Thus results from a test involving a dispersant may be specific for a defined substance/dispersant system and it may be difficult to extrapolate to other exposure scenarios or conditions. Controls containing surfactant only would distinguish possible surfactant effects but not surfactant-test substance interactions.

Water Accommodated Fractions

For complex substances, such as petroleum products, containing compounds with very low water solubilities, it is recommended to use the water accommodated fraction (WAF) approach to prepare test media since they enable the toxicity of a complex test substance to be determined whilst minimising the potential for physical effects resulting from undissolved test substance. WAFs will have to be prepared separately for each test concentration by adding the appropriate amounts of test substance (expressed as weight, volume or surface area) and cannot be prepared by dilution. This is illustrated in Appendix C together with the definition of WAFs. Solvents should only be used if all the components are fully soluble in the stock solution. A detailed description of suitable methodology have been reviewed by Girling *et al* (1992). As a general principle, the recommended minimum duration of stirring to prepare the WAFs is 24 hours. However, since the duration of mixing and energy input can influence the composition, particle size and proportion of dispersed and undispersed substance in the resulting medium, the conditions may need to be varied from case to case. The general aim should be to select mixing conditions (duration, energy input), which allow the phases to be separated after one hour of standing. The separation of the phases is usually performed by separating funnels, but if this is very time consuming, suitable physical measures like pumping, centrifugation or filtration may be applied.

It is also suggested that the interval between adjacent loading rates may need to be wider than standard in order to achieve a significant stepwise progression in the concentration of aqueous phase components.

7.1.3 Test Design

A number of factors need to be considered before determining the test design and/or technical modifications necessary for the performance of tests with difficult-to-test substances. These are: chemical stability, water solubility and purity of the test substance, its ability to produce stable dispersions, to volatilise, to adsorb, and the potential effects of light attenuation. Therefore, a number of questions (Q1-Q6, see also Fig. 4) need to be answered before a decision on the experimental design can be made:

(Q1): chemical stability

Is the substance sufficiently stable in water under the test conditions (i.e. is the parent or a degradation product environmentally relevant)?

For substances that are unstable and likely to undergo degradation in the test medium, the following guidance on whether to test the unstable "parent" substance or the breakdown products is proposed for hazard identification and classification; for risk assessment, additional testing may be required:

- If the degradation half-life in the test medium is 12 hours⁴ or greater, then the toxicity of the "parent" substance should be determined.
- For substances which react spontaneously with water (e.g. certain acid chlorides or isocyanates), it is only possible to test the decomposed products.
- For other substances, the decision on whether to test the "parent" substance or the breakdown products should be assessed case by case. If there is an indication that the "parent" substance is more toxic, then for hazard identification and labelling the toxicity of the "parent" substance should

⁴ At a half life of 12 hours, approximately 80% of the nominal concentration of the "parent" substance can be maintained in a flow-through test assuming that there are at least 6 renewals of the test solution in 24 hours. Such a half-life period is therefore consistent with test guideline requirements for the maintenance of exposure concentrations and is used as a guidance value by Whitehouse and Mallet (1993).

be determined even if exposure levels cannot be maintained within appropriate limits, e.g. $\pm 20\%$ of the initial concentrations⁵.

Technical options to maintain test substance concentrations may be possible, where the degradation mechanism is known (Table 4).

If the measured concentrations cannot be maintained within appropriate limits (see Footnote 5) using a static system, some form of medium replacement will be necessary. The type and frequency of medium replacement can be determined from a pre-stability study (Figure 3) or assessment of physico-chemical properties. Semi-static (periodic replacement of medium) or flow-through (continuous replacement) test regimes are acceptable if the test substance concentrations can be maintained within appropriate limits. In *Daphnia* tests, daily replacement of the test medium is often convenient. In fish tests, semi-static or flow-through approaches may be adopted depending upon the extent of losses experienced.

Table 4. Possible technical measures to maintain test substance concentrations

Mode of loss	Technical measures
Photolysis	<ul style="list-style-type: none"> ■ Exclusion of relevant wavelengths ■ Perform test in dark ^a ■ Medium replacement
Biodegradation	<ul style="list-style-type: none"> ■ Sterilisation of media and apparatus ■ Medium replacement
Hydrolysis	<ul style="list-style-type: none"> ■ Minimisation of aqueous contact - use of solvents for stock solutions ■ pH adjustment of stock solution ■ Medium replacement

^a Feasible for acute fish and daphnia studies only.

(Q2): water solubility

Is the substance soluble when 100 mg is added per litre of test medium?

For this report, substances not completely soluble at 100 mg/l under the test conditions are called sparingly soluble substances. For these, it is recommended that they should be tested at concentrations that do not exceed their solubility, because the principal mechanism for the uptake of substances is passive diffusion; the driving force for uptake and hence toxicity is therefore the dissolved concentration in the aqueous medium. Although the presence of excess test substance (present as particulates or droplets) may be useful to maintain saturated solutions, care must be taken to avoid indirect effects,

⁵ Limits are considered to be appropriate if they meet the need of the test program. For example, the EU requires a range of $\pm 20\%$ of the initial measured concentration for testing of well soluble substances. For testing sparingly soluble substances at very low concentrations, however, wider ranges may be appropriate.

particularly in studies with Daphnia. Therefore, it is generally recommended to separate excess material. If separation techniques, such as filtration or centrifugation are employed in the preparation of the test solution to remove excess test material, they must be validated. However, separation may not always be possible as certain substances have a tendency to form stable dispersions (see Q4).

For sparingly water soluble substances, if it is suspected that a minor more water soluble component is causing the observed toxicity, then this may be revealed by a comparison of the results of testing at levels up to 100 mg/l with removal of excess material (i.e. a WAF approach used for an essentially pure substance) on the one hand and of the results of testing at the solubility limit on the other.

(Q3): purity

Is the substance 'essentially pure' (as defined in Section 2.2)?

Tests to comply with the EU Directive (67/568/EEC) for the classification, packaging and labelling of dangerous substances should be conducted on the substance as defined in the Directive and hence the purity criterion indicated in the proposed test decision scheme is not applicable, although there may be implications for the use of the data in risk assessment (Section 7.2.2). For sparingly soluble substances, it is recommended that a nominal maximum concentration of 100 mg/l is used to prepare test solutions or water accommodated (soluble) fractions. However, higher levels may be used if required for other test purposes.

(Q4): substance forms stable dispersions

Does the substance form dispersions spontaneously under the conditions of test media preparation?

For substances forming dispersions upon stirring in water, which are difficult to separate by centrifugation or filtration, testing with excess substance present is considered to be unavoidable. Presentation of the data obtained from such tests should highlight the possible difficulties in interpretation of the results. The addition of dispersing agents to create artificial dispersions should be avoided for the reasons previously stated.

(Q5): volatilisation and adsorption

Is the substance difficult to maintain at constant exposure concentration due to losses by volatilisation or adsorption?

Possible options to overcome such losses are summarised in Table 5.

Table 5. Possible Technical Options to Maintain Test Substance Concentrations With Regards to Sorption and Volatilisation

Mode of loss	Technical measures
Sorption	■ Alternative materials (e.g. rigid plastics)
	■ Surface treatment (e.g. silanisation of glassware)
	■ Pre-conditioning of glassware with test substance
Volatilisation	■ Sealed vessels with minimal headspace
	■ Medium replacement

The type and frequency of medium replacement should be determined in a manner similar to that described under (Q1).

(Q6): light attenuation

Is the substance coloured and attenuating light?

Several recommendations have been made in order to overcome the problems of light attenuation, particularly for algal tests. Whitehouse and Mallet (1993) recommend re-inoculation of algae into fresh medium (without substance) after the end of the normal exposure period which provides additional information to the standard test in order to discriminate between an algistatic and an algicidal effect. Memmert (1994) describes another procedure involving comparison of direct exposure of the coloured test substance to that of light which is filtered through the coloured test medium. A difference in effects between the two exposure regimes may give an estimate of the inherent toxicity of the test substance. Comber *et al* (1995) have proposed a modification of the design of OECD Guideline 201 (increased light intensity, reduced solution volume and algal inoculum) which results in a reduced average light path through the test solution and an increased algal growth rate. Further studies are required to validate these approaches.

Test design decision scheme

A test decision scheme for difficult-to-test substances is outlined in Figure 4. The purpose of the scheme is to provide a logical and consistent basis for the development of a test protocol. Critical decision steps concerning the test substance are questions Q1-Q6 shown above. Question 6, however, is not incorporated in Figure 4, as it only applies to algae and *daphnia* tests requiring specific modifications.

The decisions based on Q1-Q5 lead to thirteen test scenarios (T1-T13). The implications on the use of the data for risk assessment has been noted in Table 6, and is discussed further in Section 7.2.

An initial range-finding or limit test (depending upon the expected toxicity of the test substance) may be carried out in order to determine exposure levels for the definitive tests. If a significant effect is observed then a definitive study should be performed in the corresponding concentration range. If no effect was seen, the limit test is the final study.

7.1.4 Analytical support

The Task Force endorsed most of the recommendations given by Stephenson (1992) and Whitehouse and Mallet (1993), which were integrated in this section.

Generally, analysis of solutions is carried out to determine the relationship between the toxic end point and the actual exposure concentrations, as well as to assess the stability of the exposure over the test period. For *complex substances* analysis serves additional objectives, such as to demonstrate that the duration of mixing during preparation of the WAF was sufficient to maximise the concentration of dissolved components and to assess any changes in composition of the medium.

Determination of Test Substance Concentration

Analytical method requirements

Validated analytical methods should be used for the determination of test substance concentration. Ideally, the analytical method should provide reliable results down to the lowest test concentration. The analytical blank value and the recovery of the substance as well as precision and accuracy should be known for the relevant concentration range and for the matrices concerned. If available, a substance-specific analytical method should be used. As an alternative, a method specific for a group of substances may be used (e.g. surfactants).

For *complex substances*, analysis of WAFs is usually performed by non-specific methods, as it is neither required nor practicable to quantify the exposure levels of individual compounds.

Analytical data requirements

Periodic visual observations of the test solutions should be made at intervals throughout the test to record the presence of undissolved test substance, homogeneity and changes in appearance, although the lack of a visible precipitate does not assure a true solution.

Analysis of a control (solvent control if used) is made on each occasion that samples from vessels containing test substances are taken for analysis. This may include additional controls containing the test substance but no test organisms, i.e. test medium on its own or test medium with test substance.

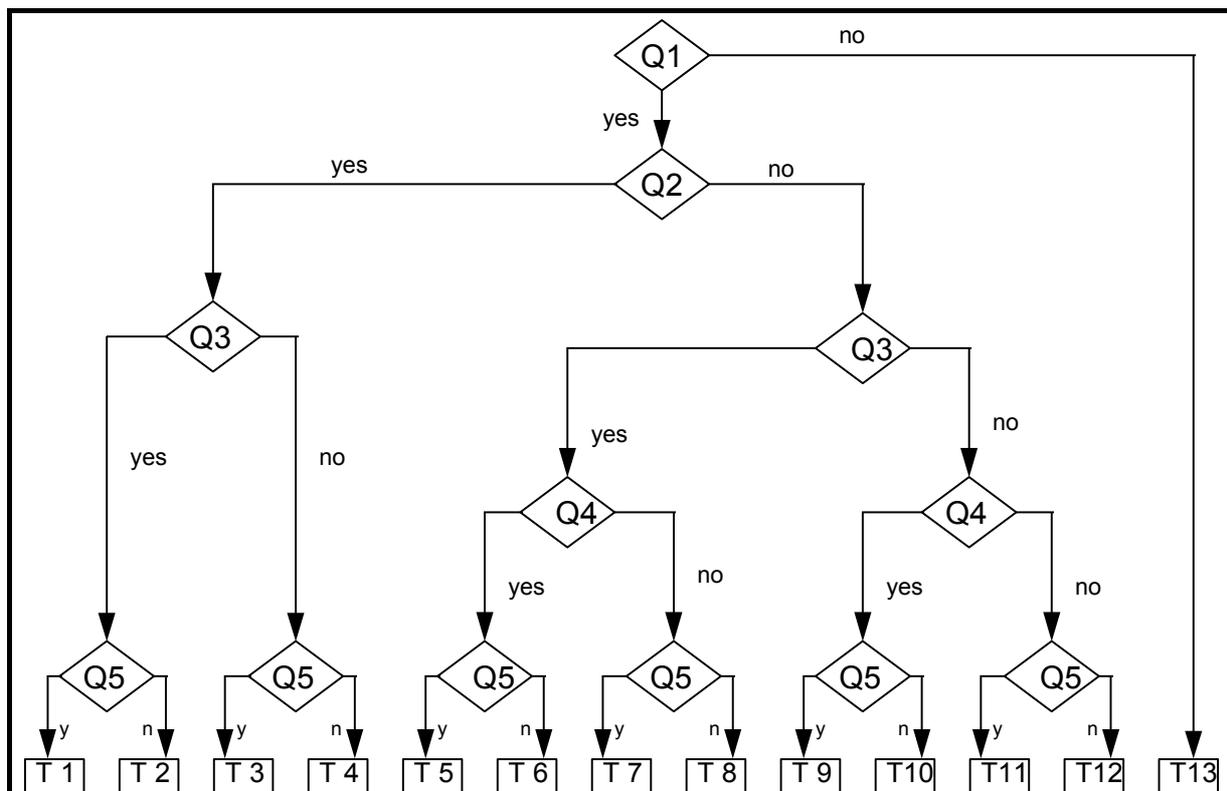
Analysis at low concentrations

It is to be expected that analysis at low concentrations will on occasions lead to greater variability in results than would be expected at higher concentrations. It may be more difficult or impossible to demonstrate stable exposure concentrations (e.g. within ± 20 % of the mean, see Footnote 5) and therefore be necessary to analyse more frequently than would be the case at higher concentrations in order to allow a reasonable estimate of the mean exposure concentration.

Sufficiently sensitive analytical methods may not be available when carrying out tests at low concentrations. In such cases analysis should be carried out at higher concentrations and estimates made as to the likely concentrations of test substance after dilution. It should be borne in mind, however, that some losses of test substance (like adsorption) may not be relative but absolute. In such cases the actual concentration may be significantly lower than the extrapolated concentration. Possible options to improve analytical sensitivity include the use of larger volumes of test medium for subsequent extraction and analysis, as well as the use of radiolabelled test substance with subsequent identification of the molecule.

When analysis is not possible at any of the exposure concentrations, evidence should be provided on the stability of the test substance in the test medium at higher concentrations including stock solutions, taking into account volatility, biodegradability, hydrolysis etc.

Figure 4: Strategy for Testing

**(Q1): chemical stability**

Is the substance sufficiently stable in water under the test conditions (i.e. is the parent or a degradation product environmentally relevant)?

(Q2): water solubility

Is the substance soluble when 100 mg is added per litre of test medium?

(Q3): purity

Is the substance 'essentially pure' (as defined in Section 2.2)?

(Q4): substance forms stable dispersions

Does the substance form dispersions spontaneously under the conditions of test media preparation?

(Q5): volatilisation and adsorption

Is the substance difficult to maintain at constant exposure concentration due to losses by volatilisation or adsorption?

Table 6: Details of Test Scenarios

Test scenario	Are technical measures required to maintain media concentrations?	Test preparation	media	Expression of exposure (Note 1)	Is caution required in the use of the data in risk assessment?
T 1	yes	series of dilutions, up to 100 mg/l		measured concentration	no
T 2	no	series of dilutions, up to 100 mg/l		measured concentration	no
T 3	yes	series of dilutions, up to 100 mg/l		a) loading or b) measured concentration of individual components	yes
T 4	no	series of dilutions, up to 100 mg/l		a) loading or b) measured concentration of individual components	yes
T 5	yes	series of dispersions up to 100 mg/l		measured soluble concentrations	yes (Note 2)
T 6	no	series of dispersions up to 100 mg/l		measured soluble concentrations	yes (Note 2)
T 7	yes	series of dilutions, up to water solubility		measured concentrations	no
T 8	no	series of dilutions, up to water solubility		measured concentrations	no
T 9	yes	series of dispersions up to 100 mg/l		loadings	yes
T 10	no	series of dispersions up to 100 mg/l		loadings	yes
T 11	yes	WAF up to 100 mg/l		loadings	yes
T 12	no	WAF up to 100 mg/l		loadings	yes
T 13	no	breakdown product(s)		concentration of loading rate of "parent" used to prepare test media	yes

Note 1: If technically feasible, exposure concentration should be determined on the soluble fraction.

Note 2: Although soluble concentrations are measured, it is possible that physical effects due to dispersed material may occur.

Sampling during the toxicity test

Sampling regimes for different types and frequencies of medium replacement

As a general guidance, in all test regimes, static, semi-static and continuous flow tests, analysis of the highest, medium and lowest test concentration at the beginning and at the end of the test is recommended as a minimum. If equivalent losses cannot be substantiated at all test concentrations, then such limited analysis is insufficient. Semi-static and continuous flow tests require additional sampling:

- semi-static: sampling at least twice (start and end of renewal period) and at regular intervals for tests lasting several weeks.
- continuous flow: sampling three times during the test or three times during the first week and thereafter in regular intervals, e.g. weekly. If the stock solution is changed during the test, it might be advantageous to check the concentration of each stock solution before use.

It is recognised that in certain circumstances sampling frequencies higher or lower may be warranted, but should be justified.

Sampling of tests with low test solution volumes

If the requirement of test solution volume for analytical purposes is much higher than for toxicity test purposes (tests on daphnia and algae toxicity), it might be useful to check the stability of the test substance in separate vessels. The same might be necessary if the analytical method is not applicable with test organisms present (tests on algae or bacteria toxicity).

Sampling of Difficult-to-test substances

For *sparingly soluble substances*, significant substance losses may arise from adsorption to surfaces which come into contact with the solutions. These losses can be reduced by:

- partitioning the substance from the aqueous phase into a solvent, such as hexane, using primed sampling bottles (e.g. extraction flasks) in preparation for analysis, as most compounds which sorb onto glass are highly lipophilic.
- the use of different materials of test containers, surface treatment (silanisation) or preconditioning of the sample containers with solutions of the test substance in the concentration range to be analysed.

Samples of solutions containing *unstable substances* should be analysed immediately after sampling or they need to be stabilised.

For stable *dispersions*, whole samples should be submitted for analysis.

7.1.5 Calculation and Expression of Results

For *water-soluble substances* the results (LC/EC/NOEC-values) should be calculated from the measured exposure concentrations (initial and final). For static and semi-static tests, the results should be expressed as the geometric means⁶ of the measured concentrations. In flow-through tests it may be more appropriate to use arithmetic means. In both cases the mean values should be rounded to two significant figures, e.g. 25.3 mg/l becomes 25 mg/l.

For *sparingly soluble substances* if a dispersion is tested, then the results should be expressed in terms of loading rates (LL/EL/NOEL), which are equivalent to the nominal concentration. Alternatively, if the soluble concentration of the substance has been measured, then the results should be expressed as indicated above for a water-soluble substance.

For *complex substances* containing sparingly water soluble compounds, it is appropriate to express exposure in terms of the overall loading rate used in the preparation of the WAF or water soluble fraction (WSF) (see Appendix C), and toxicity in terms of LL/EL/NOEL values (CONCAWE, 1993). Thus:

LL₅₀ value = Lethal Loading Rate resulting in 50% mortality;

EL₅₀ value = Effective Loading Rate resulting in 50% effect.

Similarly the NOEC (No Observable Effect Concentration) becomes the NOEL (No Observable Effect Loading Rate). However, for a detailed interpretation of the results, it may sometimes be considered necessary to quantify individual concentrations of components in solution.

For *unstable substances*, the result should be expressed in terms of the concentration or loading rate of the parent substance used to prepare the test media. If the toxicity data are generated on the breakdown products and significant toxicity is observed, identification and quantification of the breakdown products and residual parent substance may be helpful in the interpretation of the findings.

⁶ If concentrations have been determined more frequently, the geometric mean is calculated by:

$$\text{mean conc} = \text{antilog} \left(\frac{1}{2(t_n - t_1)} \sum_{i=1}^{n-1} [(\log(\text{conc}_i) + \log(\text{conc}_{i+1})) \cdot (t_{i+1} - t_i)] \right)$$

where t_1 = initial time < t_2 < ... < t_n = final time;

7.2 INTERPRETATION AND USE OF DATA

Correct interpretation of effects observed in aquatic toxicity tests is dependent upon:

- test media being prepared in a manner that is appropriate to the objectives of the study and the nature of the test substance;
- correct measurement and expression of exposure levels;
- the distinction of true toxicity from indirect physical effects, such as fouling or smothering of the organism.

7.2.1 Use of Data for Hazard Identification and Classification and Labelling

In the EU, hazard identification data are used as a basis for classification and labelling of substances and the procedures are clearly defined in the relevant legislation (EEC, 1992).

If testing is performed in the context of classification and labelling of substances under the 7th Amendment to EU Directive (67/548/EEC), it is stipulated that the toxicity is determined for the registered substance (listed in EINECS or ELINCS) albeit that from a chemical perspective it may contain various chemical elements and their compounds⁷. For the purposes of classification and labelling of a substance as “dangerous to the environment” it is not relevant that, for some substances, the toxicity may be caused by components other than the named substance⁸.

For all difficult-to-test substances, if a meaningful test can be designed by selecting appropriate test conditions as indicated in Section 7.1.3, the data can be used for classification and labelling. If sparingly water soluble substances are toxic at a concentration below their water solubility then they should be classified according to the Directive. However, for sparingly soluble substances which in acute tests do not display toxicity at the water solubility limit and toxic impurities are not present in significant amounts, the toxicity criterion for classifying a substance as “dangerous to the environment” is not fulfilled. For substances which can only be tested using dispersions or WAFs, the results expressed in loading rates should be considered to be equivalent to the standard EC/LC values and the substances classified accordingly.

conc_1 = initial concentration, $\text{conc}_2, \dots, \text{conc}_n$ = final concentration.

⁷ In this report, two diverging cases of a substance have been defined, namely: an essentially pure substance with one major component and minor component(s) or impurity(ies) as well as a complex substance which is a homogeneous aggregate of a number of compounds.

7.2.2 Use of data for risk assessment

Environmental risk posed by any substance released to the environment will be due to the sum of its components. Therefore, the applicability of the test data for those substances that contain compounds with widely differing characteristics and/or toxicities needs to be assessed on a case by case basis. In certain circumstances additional testing of individual compounds and separate risks assessments may be necessary.

Certain groups of substances require special attention:

Complex substances

Toxicity data expressed in terms of loading rates cannot be used as the basis for determining PNECs. Furthermore for certain complex substances, such as oil products, it may not be feasible to test individual compounds. Instead, the following approaches may be suitable for the purpose:

- QSAR principles should be applied, where possible, and knowledge of the composition of the substance used to evaluate existing data from related substances;
- for petroleum products the so-called 'block' approach recommended by CONCAWE (1993)⁹ should be used which was accepted by the EU (EEC, 1996).

The block approach is based on the assumption of additivity which may, however, not apply for other groups of substances, or for the application of sublethal effects.

Substances not toxic at their water solubility limit

⁸ For sparingly water soluble substances, if it is suspected that a minor more water soluble component is causing the observed toxicity, then this may be revealed by a test design as described under Q2 in Section 7.1.3.

⁹ The principle of the approach is the following:

- 1.) The distribution and fate of a chemical is dependent on its physico-chemical properties and its susceptibility to degradation. In the case of a mixture, each component will be distributed and subjected to fate processes independent of the other components of the mixture. Estimations of PECs can therefore only be made for the individual components, although in reality groups of closely related chemicals, such as the isomers and adjacent members of homologous series of hydrocarbons, may be so similar in their properties that they can be grouped and treated as single compounds without introducing serious errors.
- 2.) Given that PECs can only be derived for components, it follows that PNECs must also be estimated for components, or groups of components. Therefore acute ecotoxicity data obtained with **whole** petroleum products, whether obtained using WAFs or dispersions, cannot be used to estimate PNECs.
- 3.) Given the above, it is clear that PEC/PNEC ratio of a mixture cannot be derived directly; it must be derived from the PEC/PNEC ratios of the (groups of) components. Starting from established toxicological principles and assuming additivity of effects, the PEC/PNEC ratio of a mixture can be calculated using the equation:

$$\frac{PEC}{PNEC}^{mix} = \frac{PEC_A}{PNEC_A} + \frac{PEC_B}{PNEC_B} + \frac{PEC_C}{PNEC_C} \text{ etc.}$$

where the mixture contains known amounts of components, or groups of components, A, B, C, etc.

If no toxicity value exists for a substance, then it is impossible to derive a PNEC for a risk assessment. Furthermore, even if a substance is shown to be non toxic in an acute test at its limit of solubility, it cannot be assumed that toxic effects will not occur at lower concentrations over a chronic exposure period. Therefore, the following conservative approach is proposed in order to avoid unnecessary chronic testing: The highest measured soluble concentration (or solubility limit) is taken as the acute LC_{50} and the assessment factor usually applied to the LC_{50} is assigned to that concentration in order to calculate a safe upper limit for a PNEC. Only in those cases where the risk assessment using the resulting value indicates an upper limit for the risk characterisation ratio of >1 , then it may be necessary to determine the chronic toxicity of the substance.

7.3 FUTURE RESEARCH

The discussion and literature review presented above has highlighted areas where the available information is insufficient for clear conclusions to be drawn. Some of these could benefit from further research.

The specific aspects of testing 'difficult substances' where the Task Force believes further effort should be placed are:

- investigation of methods of rapidly and reliably preparing test media in large volumes such that the maximum solubility can be obtained quickly;
- investigation of the physical chemistry of complex liquid mixtures, particularly in respect of the solubility (i.e. partitioning) of the individual components, in order to aid the development of the 'block' method for risk assessment;
- study of any relationship between dissolved concentration of substance, uptake of substance and the method of media preparation, as has been started by Marshall and van Egmond (1995);
- study of the potential of particles to cross membranes;
- development of a method for testing algae under flow-through conditions;
- study of the effects of dispersants on toxicity of substances;
- study of the acute/chronic ratios of sparingly soluble substances.

However, the direction of developments in aquatic toxicity testing will be influenced by factors such as the following.

Developments in analytical chemistry methods: these could provide many opportunities for obtaining greater insights into, for example, the toxicity of multicomponent substances. Increased sensitivity and coupling of techniques could change the extent of analysis that is possible.

Developments in testing techniques: experience with direct toxicity assessment (DTA) as a monitoring tool will provide new insights into the mechanisms of the expression of toxicity.

8. SUMMARY OF RECOMMENDATIONS

The recommendations and findings discussed and developed by the Task Force are given in the earlier sections, and may be summarised as follows:

General

- Before starting aquatic toxicity tests, the purpose or purposes for which the data are required should be known.
- There should be sufficient understanding of the nature and physico-chemical properties of the substance to design an appropriate exposure system. Guidance on what 'sufficient understanding' means is provided.

Solubility

- The solubility of a substance in water may not be the same as its solubility in the test medium, and that solubility may be difficult to attain under the conditions in which test media are prepared.
- Solubility has a specific interpretation in the context of complex substances, and is dependent on the conditions, especially the amount of substance present per unit volume of medium (the 'loading rate').

Uptake of substances

- Uptake is primarily by passive diffusion. For materials dissolved in water, this diffusion occurs across the respiratory surface. For materials in food, it occurs across the cell boundary of the digestive system.

Preparation of test medium

- Low concentrations of co-solvents of low toxicity may be used, if necessary, as a vehicle of dosing.
- Dispersants should not be used to aid preparation of medium.

Performance of tests

- Performance of toxicity tests with excess substance present should be avoided, if technically feasible; any dose-toxic response relationship obtained is probably not due to toxicity of the main components of the substance; several possible explanations for such results have been presented.
- Test methodologies to maintain exposure concentrations of volatile, adsorptive or unstable substances exist, and should be employed when appropriate.

- The use of water accommodated fractions (WAFs) as test media is recommended for the testing of complex multi-component substances. WAFs should be prepared at the required 'loading rates' separately, and should not be serially diluted.
- A test design scheme is presented, based on consideration of the properties of the substance, and advice on the correct interpretation of the data is given.
- For tests with essentially pure substances, the results (LC/EC-, NOEC-values) should usually be calculated from exposure levels expressed as the geometric means of the measured concentrations of the dissolved substance. For complex multi-component substances, loading rates used to prepare the WAFs are used; for dispersions loading rates should also be used.
- The advice regarding analytical chemistry aspects of testing, and the scheme for choice of test design, should be considered by those setting up studies.

Guidelines

- Regulatory guidelines and methods for aquatic toxicity testing were developed for essentially pure substances that are soluble and stable in water, and consequently require modification to properly account for substances which are difficult to test.

Risk assessment

- In an initial risk assessment for substances that are not acutely toxic at their water solubility limit, the following conservative approach is proposed in order to avoid unnecessary chronic testing: The highest measured soluble concentration (or solubility limit) is taken as the acute LC₅₀ and the assessment factor usually applied to the LC₅₀ is assigned to that concentration in order to calculate a safe upper limit for a PNEC.

Classification and labelling

- If sparingly water soluble substances are toxic at a concentration below their water solubility then they should be classified according to the Directive. However, for sparingly soluble substances which in acute tests do not display toxicity at the water solubility limit and toxic impurities are not present in significant amounts, the toxicity criterion for classifying a substance as "dangerous to the environment" is not fulfilled.

APPENDIX A. MEASUREMENT AND CALCULATION OF SOME PHYSICO-CHEMICAL PROPERTIES AND THEIR LIMITATIONS RELEVANT TO ECOTOXICITY TESTING

The measurement of physicochemical properties has been described in detail in EEC (1992) and OECD (1992) publications. This Appendix does not attempt to repeat that information, but merely puts it in the context of difficult-to-test substances.

The estimation of physico-chemical properties by calculation or experimental methods is an important aid to the physical chemist. Values that are sufficiently accurate for the purposes of providing information prior to ecotoxicology testing may frequently be accessible by these methods. They usually require the knowledge of the chemical composition and the structure of the substance. The book edited by Lyman *et al* (1982) is an excellent source for these purposes, and a review by Fisk (1995) covers recent developments. ECETOC has established a task force which deals with various aspects of (quantitative) structure-activity relationships (QSARs) in ecotoxicology (ECETOC Technical Report, in preparation).

A.1 SOLUBILITY OF SUBSTANCES IN WATER

Definition of solubility

For test substances which contain more than one component, the definition of solubility has to be amended. For solids, the individual components may achieve different saturated solution concentrations than they would if pure. This occurs because the crystalline form of the substance (i.e. the mixture) may be different from that of any of the pure components¹⁰.

For liquid mixtures, the position is quite different. When a liquid mixture, e.g. of hydrocarbons, is placed into water, there is a multiple partitioning process. For example, consider hexane present in a multicomponent mixture. The concentration of hexane found in water will depend upon:

- the mole fraction in the mixture;
- the volume ratio;

¹⁰ In a mixture of pure crystalline components, which do not form mixed crystals, the solubilities of the components remain the same as for the single components. However, deviations may occur, when the high solubility of one component changes the polarity of the solvent and, thus, influences the solubility of the other(s), mostly in the form of a solubilisation. In the case of the formation of mixed crystals, which is common when mixtures are prepared from melts, these mixed crystals have different solubilities than the pure crystals.

- the partition coefficient between water and the mixture.

Thus the maximal "solubility" of hexane in water from the mixture can be very much lower than its true solubility as a single substance. Peterson (1994) has described this for mixtures of hydrocarbons.

Methods for measuring solubility

The methods for determining water solubility were developed for pure substances (not for technical products containing impurities) and are described in the guidelines of the OECD (1992) and the EEC (1992):

- the shake flask method;
- the column elution method.

Both have limitations depending on the physico-chemical properties of the substance in question. Care should be taken in extrapolating solubilities determined in standard methods to that which can be achieved in the toxicity test media, because there are differences in media composition.

The methods involve production of a saturated solution, separation of non-dissolved test substance and determination of the concentration of the substance in the resulting solution. The methods do not apply to volatile substances.

Shake flask method

An excess of substance is added to water and the mixture is agitated by stirring or shaking. Care should be taken to avoid the formation of a third phase (e.g. an emulsion).

It is possible to carry out the agitation stage at a temperature slightly higher than that for final equilibration. The necessary agitation time may be long and it is recommended to carry out several analyses while increasing the time interval, until a steady concentration is achieved. Hague and Schmedding (1975) have shown for five polychlorinated biphenyls that this equilibrium might take one month to be reached (an extreme case).

Elimination of the excess of substance can be obtained either by filtration or by centrifugation. It is necessary to ensure that the filter material does not adsorb any of the dissolved substance, which may be achieved by choice of the filter material or by pre-saturation of the latter with the substance.

Centrifugation is less subject to adsorption phenomena, but the degree of separation depends on its intensity and should be determined on a case-by-case basis. The effect depends on the density and the

apparent diameter of the particles to be separated. Biggar and Riggs (1974) have given the results of apparent solubility (that which appeared to be dissolved) for organochlorine pesticides according to the intensity of the centrifugation, by calculating, according to Stokes' rule, the maximal diameter of colloidal particles separated. From 0.01 μm to 0.05 μm the solubility varied by a factor of 5.

In the case of substances of solubility <10 mg/l, the flask method with direct addition of substance is not advisable, mainly due to the difficulties of separation of the excess substance.

However, various authors have proposed alternative shake-flask methods for sparingly soluble substances, which have similarities to the column method: for instance, deposition of the substance along the wall of the vessel from a solution in a volatile solvent, which is then removed (Hague and Schmedding, 1975). Alternatively, deposition onto glass beads which are then shaken with water (Hashimoto *et al*, 1982) has given results comparable to those obtained with the column elution method. The deposit on the wall may also be obtained by sublimation (Marple *et al*, 1986).

Column Elution Method

The test substance is laid on the surface of glass beads (Friesen *et al*, 1985; Dunnivant and Elzerman, 1988) or on Chromosorb W (Dickhut *et al*, 1986; Doucette and Andren, 1988). The coated material is packed into an elution column of liquid chromatography type made of steel (De Voe *et al*, 1981; Dunnivaut and Elzerman, 1988; EPA standard) or glass (Dickhut *et al*, 1986; Doucette and Andren, 1988; OECD, 1992). A flow of water through the column is then established, either with or without recirculation. A single pass has the advantage that it is possible to couple the column directly to a HPLC analysis system through an extraction column (De Voe *et al*, 1981). It is also normal with this method to run the experiments a number of times at differing flow rates. In this way the contact time of the water with the substance is varied, and it may be established if a true equilibrium has been set up.

Caution must be applied when using the column elution method with mobile liquids because the substance can be washed off the column.

A.2 MEASUREMENT OF ACID DISSOCIATION CONSTANT

Knowledge of the acid dissociation constant (K_a) is important because ionisation changes will always be accompanied by significant changes in solubility. Thus, an acid with a pK_a of 6 will dissolve perhaps to a mg/l level at pH 5, but to a g/l level at pH 7. For measurement of K_a , the OECD guideline is useful, but the book by Albert and Serjeant (1971) includes more methods and detail. The methods are applicable to mixtures of similar components, but are difficult to apply to more chemically diverse mixtures.

A.3 MEASUREMENT OF HYDROLYTIC, OXIDATION AND PHOTODEGRADATION RATES

For hydrolysis the OECD guideline is recommended, although in the context of ecotoxicity testing the pH used could be restricted to one or two values in the pH range of 6 to 9. Estimation of the rate of hydrolysis has been reviewed (Neely, 1985). Testing of the rate of oxidation follows the same principles as for hydrolysis, but should use constantly aerated solutions.

The measurement of the rate of photodegradation is best conducted using light conditions that are relevant to the aquatic toxicity testing, i.e. solar simulation or artificial light as necessary. The best available principles of testing are the EPA Fate Guidelines 161-2, although ECETOC (1984) is valuable for studies intended to gain a comprehensive insight into environmental fate.

A.4 MEASUREMENT OF OCTANOL/WATER PARTITION COEFFICIENT

Measurement of partition coefficients has been extensively described (e.g. ECETOC, 1996). However, it is relevant to note that the method of estimation of K_{ow} by HPLC is particularly useful for components of complex substances (OECD method 117); it is important also to stress that surfactants are excluded from consideration for this property.

A.5 MEASUREMENT OF VAPOUR PRESSURE AND VOLATILISATION

It is of great importance in the design of aquatic toxicity tests (for media preparation and test performance) to know the volatility of the substance under test. When no excess test substance is present, the partition of the substance between water and air (Henry's constant) is important; if excess substance is present, then the vapour pressure is also important. However, Henry's constant can be estimated from the ratio of vapour pressure to water solubility.

In sealed laboratory test systems, application of simple physical chemistry principles of mass balance gives relationships to describe the extent of loss of volatile substances to the vapour phase. With respect

to laboratory test systems that are open, the rate of volatilisation is much more difficult to assess. This subject has been reviewed (Thomas, 1982; Mackay, 1985). In practical terms, they demonstrate that a Henry's constant of $0.1 \text{ Pa m}^3 \text{ mol}^{-1}$ will give rise to a loss of substance at rates that are important relative to the length of typical tests.

Vapour pressure

Vapour pressures of substances range over at least 11 orders of magnitude. It is therefore not surprising that many techniques are known for measurement of vapour pressure. The EEC guidelines are particularly useful for describing the range of applicability of each method. In the case of complex substances, great care has to be taken. Some comments are given:

Gas saturation method	Should not be used for mixtures.
Static method	Useful, but care should be taken to ensure that the data are not distorted by volatile impurities.
Effusion methods	It is essential to leave the system long enough to ensure that a consistent result is obtained, not influenced by volatile impurities.

Estimation of vapour pressure from gas chromatographic retention time has been described (Hinckley *et al*, 1990). It can be accurate to within an order of magnitude and is therefore a useful approach, and is more appropriate than estimates from calculations. The most reliable calculation methods, such as the modified Watson method (Grain, 1982) require the input of the boiling point of the substance, which is frequently not available. The Stein and Brown method (US-EPA, 1985) to estimate the boiling point is useful.

Henry's Constant

The measurement of Henry's constant directly should rarely be necessary for substances of vapour pressures less than 1,000 Pa and solubilities less than 1 molar. Estimation of this property from the ratio of vapour pressure to water solubility is reliable. However, there may be difficulties for some complex substances, each component of which has its own Henry's constant. The vapour pressure data available may reflect the high values associated with minor components. There are calculation methods available, such as the program HENRY (Meylan, 1991) which is useful provided the test substance contains functional groups which it recognises.

APPENDIX B. DEFINITION AND ANALYSIS OF COMPLEX SUBSTANCES

For complex substances it is necessary to consider whether the individual components present should also be included in any chemical analysis. Distinguishing substances consisting of one major component containing minor components (impurities) from complex substances is a matter of judgement based on the percentage present: there is as yet no formal regulatory guidance as to how much of a minor organic component one can have in a commercial substance for it still to be considered as an "impurity". Scientifically, any component which contributes to the toxicity of the mixture needs to be considered. The following is a possible approach.

Consider for example, a substance which consists of mainly one component, but substantial amounts of a second (e.g. 3:1). What approach should be taken to determination of the amount of substance dissolved in a water solubility test? The two possibilities would be as follows:

- (a) Where, as is quite often the case, a technique such as HPLC or GC is used to analyse the amount of substance dissolved, then it should be simply stated that, in the case of the water-solubility, for example, 15.5 mg/l of the major and 3.5 mg/l of the minor component were present in the clear solution obtained following the particular water solubility determination. However, this leads to several problems:
- Because there is excess undissolved substance in the standard test methods (overtly in the flask procedure, on the support in the column procedure) it will be the more soluble component which will dissolve preferentially, by definition, resulting in different amounts of excess substance for each component. Chemical analysis could establish to what extent there had been preferential dissolution of the more soluble component. In particular, for liquid mixtures, the amount of substance dissolved will depend on the 'loading rate' up to the point of saturation.
 - The interactions between the components means that the observed solubilities will differ from the true values of the individual components, but this is not usually important.
 - In the case of substances for which it is not possible to quantify both components, a judgement of whether the analysis is acceptable has to be made.
- (b) Where the assay method is simply based on, e.g. the UV or visible absorption of the substance and all components thereof have similar such spectra, it is reasonable to present a combined

result. Thus, using the above example, one would have 19.0 mg/l as being the total amount of the two components dissolved. In the ecotoxicity test, matters may be more complicated: there would be no problem if the assay showed no degradation. However if one or both of the components degraded during the test period, this might have to be investigated using a more rigorous analytical method, like HPLC. A method of analysis which is non-separative may offer advantages for multicomponent mixtures of essentially similar substances, such as a petroleum fraction.

Some specific examples:

- (a) For closely structurally-related azo dyes, it is often adequate simply to estimate the total such species dissolved by measuring the overall UV/visible absorption. For mixtures of other closely related substances, one can sometimes use as a "handle" for the mixture a common element such as P or Si and measure that by AAS (e.g. a fire retardant consisting of a mixture of closely related phosphates).
- (b) If the substance displays very low solubility, a limit value based on, e.g. TOC or UV/visible absorption will usually suffice: e.g. less than 0.1 mg/l of total components dissolved so that it is largely immaterial what the individual solubilities are. This approach may not extend to analysis of ecotoxicology media, where the background level of organic and inorganic carbon can be significant.
- (c) In many other cases, the analytical methods available such as HPLC and/or GC may be used to give the individual solubility value of the components in the mixture.
- (d) For very complex substances, it is possible to use an incremental method to obtain water solubility: add, equilibrate, add more, etc., until no more will dissolve, and record total amount solubilised.

Therefore, the recommendations regarding analytical characterisation of the solute are as follows:

- Where one component is present at more than 90%, attempt to quantify all components present at 3% or greater, within reasonable time and cost constraints.
- Where one component is present at more than 50% but less than 90%, attempt to quantify all components present at 10% or greater, within reasonable time and cost constraints.
- In other cases, or when specific analysis is impossible, consider the use of a non-specific analytical method and present an overall result.

APPENDIX C. WATER ACCOMMODATED FRACTIONS

A Water Accommodated Fraction (WAF) is an aqueous medium containing only that fraction of a substance which remains in the aqueous phase once any source of mixing energy has been removed and after a period sufficient for phase separation. The product may be present either in true solution or as a stable emulsion (Girling, 1989; CONCAWE, 1993). CONCAWE (1993) describes the relationship of the WAF and the "Water Soluble Fraction" (WSF) in detail; essentially, the WSF consists of the component(s) of the WAF that is (are) in true solution. It also outlines the advantages of the WAF approach compared to the preparation of dispersions for testing complex substances. CONCAWE define dispersions by the requirement of either an input of energy (e.g. stirring) or the use of a chemical dispersing agent to maintain the distribution of the complex substance in the test medium. The latter deviates from the Task Force's recommendation not to use dispersing agents in aquatic toxicity tests.

The use of water accommodated fractions is appropriate to the testing of complex substances, since they enable the toxicity of a complex test substance to be determined whilst minimising the potential for physical effects resulting from undissolved test substance.

WAFs will have to be prepared for each test concentration and cannot be prepared by dilution. This is illustrated by the following examples:

Consider a two component liquid mixture, for which analytical data show the following results:

(1)	Component A	Component B
Achieved concentration at 1,000 mg/l loading	10 mg/l	1 mg/l

Should this WAF be diluted then the following would be obtained:

(2)	Component A	Component B
Tenfold dilution of (1)	1 mg/l	0.1 mg/l

However, if a WAF at 100 mg/l loading was prepared, it is quite probable that the achieved concentration would be as follows:

(3)	Component A	Component B
Achieved concentration at 100 mg/l loading	8 mg/l	0.5 mg/l

This demonstrates that for mixtures, the achieved soluble concentrations are loading dependent. Thus, the process of dilution produced a very different composition from the WAF made at lower loading, and is not acceptable.

The conditions of preparation of the WAF need to be recorded in detail and should include:

- description of substance tested;
- type of medium prepared (WAF or dispersion);
- type of mixing system;
- mass of test material and medium;
- geometry of mixing vessel;
- volume of headspace;
- method of mixing, including rotor speed, etc.;
- duration of mixing and settling/separation;
- method of phase separation in WAF preparation;
- water quality (pH, hardness, salinity, temperature) at start and end of test;
- oxygen concentration, at start and end of test;
- analytical procedure and results.

APPENDIX D. MECHANISMS FOR THE TRANSPORT OF SUBSTANCES ACROSS MEMBRANES

Passive diffusion

Most chemicals cross membranes by passive diffusion along a concentration gradient, mainly through the lipid domain of membranes.

Small hydrophilic chemicals such as ionised organic acids and bases usually have low lipid solubility. Hence, passive uptake is mainly restricted to aqueous pores and is relatively slow (Benz *et al*, 1980).

Active transport

Active transport systems show four characteristics. Such systems will:

- move the chemical against an electrochemical or concentration gradient;
- saturate at high substrate concentration;
- be selective for certain structural features of the chemical, hence it has the potential for competitive inhibition between substances to be transported by the same transporter;
- require expenditure of energy.

An example of active transport is the ATP-driven uptake of K^+ by the Na-K ATPase. Another example of active uptake via a carrier is assumed for 2,4-dinitrophenyl acetic acid (2,4-D) and indolyl acetic acid (IAA) (Donaldsen *et al*, 1973). Very few examples have been documented which are relevant to xenobiotics, and none which relate to the concerns of this Task Force.

Facilitated diffusion

Facilitated diffusion is a carrier mediated transport process that saturates at high substrate concentration and is selective for certain chemical structural features. However, this mechanism does not move chemicals against an electrochemical or concentration gradient, and it does not expend energy. An example of facilitated diffusion is glucose uptake in the intestine.

Pinocytosis and phagocytosis (particle uptake)

Observations that whole body immersion and oral immunisations in fish can be effective in eliciting antibody responses (Rodgers and Austin, 1985; Lamers and De Haas, 1985; Mughal and Manning, 1985) suggests that uptake of antigens occurs via pinocytosis and phagocytosis. Since antigens are large molecules with a low water solubility the uptake of the other types of types of particulate substances via pinocytosis and phagocytosis is possible. Few papers dealing with this subject are found, therefore there is currently no evidence for these being important processes in aquatic toxicity testing.

The intercellular uptake of kaolin clay and other suspended mineral particles by phagocytosis at the gills and deposited in the spleen of fish is described by Goldes *et al* (1986) and Martens and Servizi (1993). Hockney (1985) suggests that the gill and digestive tract may have a greater capacity for particulate uptake than the skin of fish.

Absorption of particulates in the stomach and distribution throughout the body via the blood system has been reported in mammals (Klaassen, 1980).

However, the processes by which these particulates and also fat are taken up in the intestine are not well understood (Patton, 1986), but it has been demonstrated that uptake requires solubilisation and diffusion of soluble compounds across the intestinal membrane (Van Veld, 1990; Gobas *et al*, 1993). This means that the final step to reach the target is passive diffusion.

BIBLIOGRAPHY

- Adema, D.M.M., 1980. Determination of toxicity. In: Degradability, ecotoxicity and bio-accumulation. Government Publishing Offices, The Hague, Netherlands, pp. 156-253.
- Albert A and Serjeant EP, 1971. The determination of ionization constants, Chapman and Hall.
- Anliker R, Moser P and Poppinger D (1988). Bioaccumulation of dyestuffs and organic pigments in fish. Relationships to hydrophobicity and steric factors. *Chemosphere*, 17, 1631-1644.
- Ariens EJ, 1971. A general introduction in the field of drug design. In: Ariens EJ (ed), *Drug design* Vol. 1, Academic Press, New York, pp. 2-41.
- Bahadir M, Parlar H and Spiteller M (eds), 1995. *Springer Umweltlexikon*, Springer-Verlag, Berlin, pp. 288-289.
- Barahona-Gomariz, M.V., Sanz-Barrera, Sanchez-Fortun S., 1994. Acute toxicity of organic solvents on *Artemia salina*. *Bull., Environ. Contam. Toxicol.* 52, 766-771.
- Benz R, Janko K and Läuger, P, 1980. Pore formation by the matrix protein (porin) to *Escherichia coli* in planar bilayer membranes. *Ann.N.Y.Acad.Sci.* 358, 13-24.
- Berglund R and Dave G, 1981. Influence of co-solvent (Acetone) and ultrasonication on the acute toxicity of a quaternary amine (Aliquat 336) and an organophosphorous compound (HDEHP) to *Daphnia magna*. *Bull. Environm. Contam. Toxicol.* 27, 316-325.
- Biggar JW and Riggs RL, 1974. Apparent solubility of organochlorine insecticides in water at various temperatures. *Hilgardia* 42, 383-391.
- Black MC and McCarthy JF, 1988. Dissolved organic macromolecules reduce the uptake of hydrophobic organic contaminants by the gills of rainbow trout (*Salmo gairdneri*). *Environ.Toxicol.Chem.*, 7, 593-600.
- Bowman, M.C., Oller, W.L. and T. Cairns, 1981. Stressed bioassay systems for rapid screening of pesticide residues. Part I: Evaluation of bioassay systems, *Arch. Environm. Contam. Toxicol.* 10, 9-24.
- Bringmann G. and R. Kühn, 1982. Ergebnisse der Schadwirkung wassergefährdender Stoffe gegen *Daphnia magna* in einem weiterentwickelten standardisierten Testverfahren. *Z. Wasser Abwasser Forschg.* 15, 1-6.
- Bringmann, G., and R. Kühn, 1976. Vergleichende Befunde der Schadwirkung wassergefährdender Stoffe gegen Bakterien (*Pseudomonas putida*) und Blaualgen (*Microcystis aeruginosa*), *NWF - Wasser / Abwasser*, 117, H. 9.
- Calleja MC and Persoone G, 1993. The influence of solvents on the acute toxicity of some lipophilic chemicals to aquatic invertebrates. *Chemosphere* 26, 2007-2022.
- Comber MHI, Smyth DV, Thompson, RS, 1995. Assessment of the toxicity to algae of coloured substances. *Bull. Environ. Contam.Toxicol.* 55, 922-928
- CONCAWE, 1993. Ecotoxicological testing of petroleum products: Test methodology. Report No. 92/56, CONCAWE, Brussels, Belgium.
- De Voe H, Miller MM and Wasik SP, 1981. Generator column and high pressure liquid chromatography for determining aqueous solubilities and octanol-water partition coefficients of hydrophobic substances. *J. Res. Nat. Board Stand.* 86, 361-366.
- Dickhut RM, Andren AW and Armstrong DE, 1986. Aqueous solubility of six PCBs. *Environ. Sci. Technol.* 20, 807-810.
- Donaldson TW, Bayer DE and Leonard OA, 1973. Absorption of 2,4-D and 3-(p-chlorophenyl)-1,1-dimethylurea (monuron) by barley roots. *Plant Physiol.* 52, 638-645.

- Doucette WJ and Andren AW, 1988. Aqueous solubility of selected biphenyl, furan and dioxin congeners. *Chemosphere* 17, 243-252.
- Dunnivant and Elzerman, 1988. Aqueous solubility and Henry's law constant data for PCB congeners for evaluation of quantitative structure property relationships. *Chemosphere* 17, 525-541
- ECETOC, 1984. The phototransformation of chemicals in water: results of a ring test. Technical Report No. 12.
- ECETOC, 1992. Estimating environmental concentrations of chemicals using fate and exposure models. Technical Report No. 50.
- ECETOC, 1993a. Environmental hazard assessment of substances. Technical Report No. 51.
- ECETOC, 1993b. DHTDMAC: - Aquatic and terrestrial hazard assessment. CAS No. 61789-80-8. Technical Report No. 53.
- ECETOC, 1994a. Assessment of non-occupational exposure to chemicals. Technical Report No. 58.
- ECETOC, 1994b. Environmental exposure assessment. Technical Report No. 61.
- ECETOC, 1996. The role of bioaccumulation in environmental risk assessment: the aquatic environment and related food webs. Technical Report No. 67.
- Edwards DA, Luthy RG and Liu Z, 1991. Solubilization of polycyclic hydrocarbons in micellar nonionic surfactant solutions. *Environ. Sci. Technol.* 25, 127-133.
- Edwards DA, Liu Z and Luthy RG, 1992. Interactions between nonionic surfactant monomers, hydrophobic organic compounds and soil. *Wat. Sci. Technol.* 26, 147-158.
- EEC, 1992. Council Directive 92/32/EEC of 30 April 1992 amending for the 7th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of substances ("7th Amendment"). Off J of the European Communities L 154/1.
- EEC, 1993a. Council Regulation 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances. Off J of the European Communities L 84/1.
- EEC, 1993b. Technical guidance documents in support of the risk assessment directive (93/67/EEC) for substances notified in accordance with the requirements of Council Directive 67/548/EEC. Brussels.
- EEC, 1994. Technical guidance documents in support of the Commission Regulation (EC) No. 1488/94 on risk assessment for existing substances in accordance with Council Regulation 793/93. Brussels.
- EEC, 1996. Technical guidance documents for new and existing substances. Edited by DG ECB, Ispra, Italy.
- EPA, 1982. Environmental Protection Agency Guidelines and support documents for environmental effects testing: Technical support document for fish early life stage toxicity testing. Bureau of national affairs, Inc., Washington D.C. 20037, CHM-54, 31:5175-31:5188, pp. 29-42
- Fisk, 1995. Estimation of physicochemical properties: theoretical and experimental approaches, in *Environmental Chemistry of Pesticides*, Chapter 1. Eds. TR Roberts and PC Kearney, Wiley.
- Friesen KJ, Sarna LP and Webster GRB, 1985. Aqueous solubility of polychlorinated dibenzo-p.dioxins determined by high pressure liquid chromatography. *Chemosphere* 14, 1267-1274.
- Girling AE, 1989. Preparation of aqueous media for Aquatic Toxicity testing of oils and oil-based products: A review of the published literature. *Chemosphere* 19, 1635-1641.
- Girling AE, Markarian RK and Bennett D, 1992. Aquatic Toxicity testing of oil products - Some recommendations. *Chemosphere* 24, 1469-1472.

- Gobas FAPC, McCorquodale JR and Haffner GD, 1993. Intestinal absorption and biomagnification of organochlorines. *Environmental Toxicology and Chemistry* 12, 567-576.
- Goldes SA, HW Ferguson, Douust PY and Moccia RD, 1986. Phagocytosis of the inert suspended clay kaolin by the gills of rainbow trout (*Salmo gairdneri* Richardson). *Fish Dis J* 9, 147-152.
- Grain CF, 1982. Chapter 14, 'Vapour pressure' in Lyman WJ, Reehl WF, Rosenblatt DH, 'Handbook of Chemical Property Estimation Methods', McGraw-Hill, New York.
- Hague R and Schmedding D, 1975. A method of measuring the water solubility of hydrophobic chemicals: solubility of five polychlorinated biphenyls. *Bull. Environ. Contam. Toxicol.* 14, 13-17.
- Hashimoto Y, Tokura K, Osakik W, Strachan WMJ, 1982. A comparison of aqs by the flask and microcolumn methods. *Chemosphere* 11, 991-1001.
- Hawker DW and Connell DW, 1985. Relationships between partition coefficient, uptake rate constant, clearance rate constant and time to equilibrium for bioaccumulation. *Chemosphere* 14, 1205.
- Hermens JLM, Leeuwangh P, Musch A, 1984. Quantitative structure-activity relationships and mixture toxicity studies of chloro- and alkylanilines at an acute lethal toxicity level to the guppy (*Poecilia reticulata*). *Ecotox. Env. Safety* 8, 388-394.
- Herzel F and Murty AS, 1984. Do carrier solvents enhance the water solubility of hydrophobic compounds? *Bull. Environ. Contam. Toxicol.* 32, 53-58.
- Hinckley DA, Bidleman TF, Foreman WT, Tuschall JR, 1990. Determination of vapour pressures for nonpolar and semipolar organic compounds from gas chromatographic retention data. *J. Chem. Eng. Data*, 35, 232-237.
- Hockney NJ, 1985. An investigation of the skin of rainbow trout (*Salmo gairdneri* Richardson) for antigen uptake mechanisms following spray vaccination. In Manning M. and Tatner MF (eds), *Fish Immunology*, Academic Press, London, pp. 195-206.
- ISO International Organisation for Standardization, 1994. Water quality - Guidance for algal growth inhibition tests with poorly soluble organic and inorganic substances, volatile organic compounds, heavy metals and waste water. ISO/TC 147/SC 5/WG 5/N 151, draft August 1994.
- Jafvert CT, Van Hoof PL and Heath JK, 1994. Solubilization of non-ionic surfactant micelles. *Water Res.* 28, 1009-1017.
- Johnson WW and Finley TM (1980). Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. US Dept. of the Interior, Fish and Wildlife Service, Resource Publication 137, Washington DC.
- Kile DE and Chiou CT, 1989. Water solubility enhancements of DDT and trichlorobenzene by some surfactants below and above the critical micelle concentration. *Environ. Sci. Technol.* 23, 832-838.
- Klaassen CD, 1980. Absorption distribution and excretion of toxicants. In J. Doull, D. C. Klaassen, and M. O. Amdur (eds), *Casarett and Doull's Toxicology*, Macmillan Publishing Co., New York, pp. 28-55.
- Knezovich JP, Harrison FL, 1988. The bioavailability of sediment-sorbed chlorobenzenes to larvae of the midge, *Chironomus decorus*. *Ecotoxicol. Environ. Safety*, 15, 226-241.
- Lamers CHJ and MJH DeHaas, 1985. Bath vaccination and development of immunological memory in carp (*Cyprinus carpio* L.). In Manning M and Tatner MF (eds), *Fish Immunology*, Academic Press, London, pp. 231-232.
- Leblanc, G.A. and Surprenant D.C., 1983. The acute and chronic toxicity of acetone, dimethyl formamide and triethylene glycol to *Daphnia magna* (Straus). *Arch. Environ. Contam. Toxicol.* 12, 305-310.
- Leifer A, 1988. The kinetics of environmental aquatic photochemistry. ACS.

- Li A and Andren AW, 1994. Solubility of polychlorinated biphenyls in water/alcohol mixtures. 1. Experimental data. *Environ. Sci. Technol.* 28, 47-52.
- Lien GJ and McKim JM, 1993. Predicting branchial and cutaneous uptake of 2,2',5,5'-tetrachlorobiphenyl in fathead minnows (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*): Rate limiting factors. *Aquat. Toxicol.* 27, 15-32.
- Lyman WJ, Reehl WF, Rosenblatt DH, 1982. *Handbook of Chemical Property Estimation Methods*, McGraw-Hill, New York. This book has recently been republished by the American Chemical Society.
- Mackay D, 1985. Air/water exchange coefficients, in: 'Environmental Exposure from Chemicals', Eds. WB Neely, Blau GE, CRC Press, Boca Raton, Florida, pp. 91-108.
- Mackay D, 1992. *Multimedia Environmental Models*, Lewis Publishers.
- Marple L, Brunck R and Throop L, 1986. Watersolubility of 2,3,7,8 tetrachlorodibenzo-p-dioxin. *Environ. Sci. Technol.* 20, 180-182.
- Marshall SJ and Van Egmond R, 1995. *Bioavailability*. UK Department of Environment Research Contract, Final Report.
- Martens DW, and Servizi JA, 1993. Suspended sediment particles inside gills and spleens of juvenile Pacific salmon (*Oncorhynchus* spp.). *Canadian Journal of Fisheries and Aquatic Sciences*, 50, 586-590.
- McCarthy JF and Jimenez BD, 1985. Reduction in bioavailability to bluegills of polycyclic aromatic hydrocarbons bound to dissolved humic material. *Environ. Toxicol. Chem.*, 4, 511-521.
- Memmert U, 1994. Inhibition of algal growth caused by coloured test substances. Interim Report, RCC Project 460302, Oct 1994.
- Meylan WM, Howard PH, 1991. Bond contribution method for estimating Henry's law constants. *Environ. Toxicol. Chem.*, 10, 1283-1293.
- Mughal MS and Manning MJ, 1985. Antibody responses of young carp (*Cyprinus carpio*) and Grey Mullet (*Chelon labrosus*) immunized with soluble antigen by various routes. In Manning M and Tatner MF (eds), *Fish Immunology*, Academic Press, London, pp. 313-326.
- Neely WB, 1985. Hydrolysis. in: *Environmental Exposure from Chemicals*, Eds. W.B. Neely, Blau GE, CRC Press, Boca Raton, Florida, pp. 157-176.
- Nyssen GA, Miller ET, Glass TF, Quinn CR II, Underwood J and Wilson DJ, 1987. Solubilities of hydrophobic compounds in aqueous-organic solvent mixtures. *Environm. Monitoring and Assessment* 9, 1-12.
- OECD, 1992. *Guidelines for Testing of Chemicals*, Paris.
- OECD, 1993. *Guidelines for Testing of Chemicals. Section C: Effects on Biotic Systems: 202 - Part II: Daphnia, reproduction test (Draft 6/93 as prepared following Daphnia, Workshop, Sheffield, March 1993)*
- OECD, 1995. *Workshop on aquatic toxicity testing of sparingly soluble metals, inorganic metal compounds and minerals, 5-8 September 1995, Ottawa, Canada. Draft report.*
- Opperhuizen A, 1986. Bioconcentration in fish and other distribution processes of hydrophobic chemicals in aqueous environments. Ph.D. thesis. University of Amsterdam, the Netherlands.
- PARCOM, 1990. *Paris Commission Guidelines regarding harmonization of procedures of approval, evaluation and testing of offshore chemicals and drilling mud*. London.
- Patton JS, 1986. Is the microvillus bilayer freely permeable to lipophilic molecules? In F. Alvarado and C. H. Van Ois (eds.), *25 years of Research on the Brush Border*, Elsevier Press, Amsterdam.
- Peterson DR, 1994. Calculating the aquatic toxicity of hydrocarbon mixtures. *Chemosphere* 29, 2493-2506.
- Poirier SH, Knuth ML, Anderson-Buchou CD, Brooke LT, Lima AR, Shubat PJ, 1986. Comparative toxicity

of methanol and N,N-dimethylformamide to freshwater fish and invertebrates. Bull. Environ. Contam. Toxicol. 37, 615-621.

Poremba, K. (1993). Influence of synthetic and biogenic surfactants on the toxicity of water-soluble fractions of hydrocarbons in sea water determined with the bioluminescence inhibition test. Environmental Pollution, 80, 25-29.

Potter D, Clarius TM, Wright AS and Watson WP, 1994. Molecular dosimetry of DNA adducts in rainbow trout (*Oncorhynchus mykiss*) exposed to benzo(a)pyrene by different routes. Arch. Toxicol. 69, 1-7.

Rodgers CJ and Austin B, 1985. Oral immunization against furunculosis: An evaluation of two field trials. In Manning M and Tatner MF (eds), Fish Immunology, Academic Press, London, pp. 185-194.

Rosen, M.J., 1976. Surfactants and Interfacial Phenomena. John Wiley & Sons, Inc, New York.

Schrap SM, and Opperhuizen A, 1990. Relationship between bioavailability and hydrophobicity: reduction of the uptake of organic chemicals by fish due to the sorption of particles. Environ.Toxicol.Chem. 9, 715-724.

Schwarzenbach RP, 1993. Environmental Organic Chemistry. John Wiley and Sons Inc, New York, p. 443.

Servos MR, Muir, DCG and Webster GRB, 1989. The effect of dissolved organic matter on the bioavailability of polychlorinated dibenzo-p-dioxins. Aqu. Toxicol. 14, 169-184.

Shubat PJ, Poirier SH, Knuth ML and Brooke LT, 1982. Acute toxicity of tetrachloroethylene and tetrachloroethylene with dimethylformamide to rainbow trout (*Salmo gairdneri*). Bulletin of Environmental Contamination and Toxicology 28, 7-10.

Spacie A and Hamelink JL, 1985. Bioavailability. In Rand GM and Petrocelli SR (eds), Fundamentals of Aquatic Toxicology: Methods and Applications,

Hemisphere Publishing Corporation, New York, pp. 495-525.

Stephenson R, 1992. Guidance for analytical chemistry requirements in support of aquatic toxicity testing and some proposals on testing difficult materials. Report of a UK Ecotoxicity Shadow Group.

Stratton, W. and T.M. Smith, 1988. Interaction of organic solvents with the green alga *Chlorella pyrenoidosa*. Bull. Environ. Contam. Toxicol. 40, 736-742

Takahashi, I.T., Cowgill, U.M. and Murphy, P.G., 1987. Comparison of ethanol toxicity to *Daphnia magna* and *Ceriodaphnia dubia* tested at two different temperatures: static acute toxicity test results. Bull. Environ. Contam. Toxicol. 39, 229-236.

Thomas RG, 1982. Volatilization from water, in: Lyman WJ, Reehl WF, Rosenblatt DH, 1982. Chapter 15.

US-EPA, 1985. Federal Register, Vol. 50, No. 188, Sept. 27, pp. 39252-39321.

Van Veld PA, 1990. Absorption and metabolism of dietary xenobiotics by the intestine of fish. Rev. Aquat. Sci. 2, 185-203.

Versteeg DJ and Shorter SJ, 1992. Effect of organic carbon on the uptake and toxicity of quaternary ammonium compounds to the fathead minnow, *Pimephales promelas*. Envir. Toxicol. Chem., 11, 571-580.

Versteeg DJ, Feijtel TCJ, Cowan CE, Ward TE and Rapaport RA, 1992. An environmental risk assessment for DTDMAC in The Netherlands. *Chemosphere* 24, 641-662.

Whitehouse P and Mallett M, 1993. Aquatic Toxicity testing for notification of new substances. An advisory document on dealing with "difficult" substances. Report to the Chemical Notification Unit, Department of the Environment, CP 722. The Publications Unit, WRc plc., Henley Road, Medmenham, PO Box 16, Marlow, Buckinghamshire SL7 2HD, U.K.

MEMBERS OF THE TASK FORCE

H. Rufli, Chairman	CIBA-GEIGY CH - Basel
P. Fisk	SHELL RESEARCH UK - Sittingbourne
A. Girling*)	SHELL RESEARCH UK - Sittingbourne
J.M.H. King	UNILEVER RESEARCH UK- Wirral
R. Länge	SCHERING D - Berlin
X. Lejeune	CHEMIE ET ECOLOGIE F - Versailles
N. Stelter	HENKEL D - Düsseldorf
C. Stevens	DOW CORNING EUROPE B - Brussels
P. Suteau	RHÔNE-POULENC F - Sophia Antipolis
J. Tapp*)	ZENECA UK - Brixham
J. Thus	SOLVAY DUPHAR NL - Weesp
D.J. Versteeg	PROCTER & GAMBLE USA - Cincinnati
H.J. Niessen, Secretary	ECETOC B - Brussels

*) Corresponding Members

MEMBERS OF THE SCIENTIFIC COMMITTEE

(Peer Review Committee)

W.F. TORDOIR (Chairman), Group Adviser, Environmental Health and Human Toxicology	SHELL INTERNATIONAL NL - Den Haag
H. VERSCHUUREN (Vice-Chairman) Head, Toxicology Department	DOW EUROPE CH - Horgen
O.C. BØCKMAN, Scientific Adviser	NORSK HYDRO N - Porsgrunn
N.G. CARMICHAEL ^a , Toxicology Director Worldwide	RHÔNE-POULENC F - Sophia Antipolis
C. D'HONDT, Head of Ecology Department	CIBA-GEIGY CH - Basel
H. DE HENAU, European Technical Centre, Professional and Regulatory Services	PROCTER AND GAMBLE B - Strombeek-Bever
A. DE MORSIER ^a , Head, Chemicals Legislation Services	CIBA-GEIGY CH - Basel
P.A. GILBERT, Head, Environmental Division	UNILEVER UK - Port Sunlight
B. HILDEBRAND, Director, Experimental Toxicology	BASF AG D - Ludwigshafen
J.R. JACKSON, Director, Medicine and Health Science	MONSANTO EUROPE B - Louvain-la-Neuve
E. LÖSER, Head, Institute of Industrial Toxicology	BAYER D - Wuppertal
R. MILLISCHER, Head, Industrial Toxicology Department	ELF ATOCHEM F - Paris
I.F.H. PURCHASE, Director, Central Toxicology Laboratory	ZENECA GB - Macclesfield
G. RANDALL, Director, Brixham Environmental Laboratory	ZENECA GB - Brixham
H.J. WIEGAND, Head of Product Safety Department	HÜLS D - Marl

^a Stewards responsible for primary peer review

Reponsible Editor: FM Carpanini, ECETOC
Av E Van Nieuwenhuyse 4 (Bte 6)
B - 1160 Brussels, Belgium
D-1996-3001-137