ANNEX 9

GUIDANCE ON HAZARDS TO THE AQUATIC ENVIRONMENT

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GUIDANCE ON HAZARDS TO THE AQUATIC ENVIRONMENT [1]

A9.1   Introduction

A9.1.1   In developing the set of criteria for identifying substances hazardous to the aquatic environment, it was agreed that the detail needed to properly define the hazard to the environment resulted in a complex system for which some suitable guidance would be necessary. Therefore, the purpose of this document is twofold:

(a)   to provide a description of and guidance to how the system will work;

(b)   to provide a guidance to the interpretation of data for use in applying the classification criteria.

A9.1.2   The hazard classification scheme has been developed with the object of identifying those chemical substances that present, through the intrinsic properties they possess, a danger to the aquatic environment. In this context, the aquatic environment is taken as the aquatic ecosystem in freshwater and marine, and the organisms that live in it. For most substances, the majority of data available addresses this environmental compartment. The definition is limited in scope in that it does not, as yet, include aquatic sediments, nor higher organisms at the top end of the aquatic food-chain, although these may to some extent be covered by the criteria selected.

A9.1.3   Although limited in scope, it is widely accepted that this compartment is both vulnerable, in that it is the final receiving environment for many harmful substances, and the organisms that live there are sensitive. It is also complex since any system that seeks to identify hazards to the environment must seek to define those effects in terms of wider effects on ecosystems rather than on individuals within a species or population. As will be described in detail in the subsequent sections, a limited set of specific properties of chemical substances have been selected through which the hazard can be best described: aquatic toxicity; lack of degradability; and potential or actual bioaccumulation. The rationale for the selection of these data as the means to define the aquatic hazard will be described in more detail in Section A9.2.

A9.1.4   The application of the criteria is also limited, at this stage, to chemical substances. The term substances covers a wide range of chemicals, many of which pose difficult challenges to a classification system based on rigid criteria. The following sections will thus provide some guidance as to how these challenges can be dealt with based both on experience in use and clear scientific rationale. While the harmonized criteria apply most easily to the classification of individual substances of defined structure (see definition in Chapter 1.2), some materials that fall under this category are frequently referred to as "complex mixtures". In most cases they can be characterized as a homologous series of substances with a certain range of carbon chain length/number or degree of substitution. Special methodologies have been developed for testing which provides data for evaluating the intrinsic hazard to aquatic organisms, bioaccumulation and degradation. More specific guidance is provided in the separate sections on these properties. For the purpose of this Guidance Document, these materials will be referred to as "complex substances" or "multi-component substances".

A9.1.5   Each of these properties (i.e. aquatic toxicity, degradability, bioaccumulation) can present a complex interpretational problem, even for experts. While internationally agreed testing guidelines exist and should be used for any and all new data produced, many data usable in classification will not have been generated according to such standard tests. Even where standard tests have been used, some substances, such

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[1]   *OECD Environment, Health and Safety Publications, Series on Testing and Assessment, No 27, Environment Directorate, Organization for economic Co-operation and Development, April 2001.*

as complex substances, hydrolytically unstable substances, polymers etc, present difficult interpretational problems when the results have to be used within the classification scheme. Thus data are available for a wide variety of both standard and non-standard test organisms, both marine and freshwater, of varying duration and utilizing a variety of endpoints. Degradation data may be biotic or abiotic and can vary in environmental relevance. The potential to bioaccumulate can, for many organic chemicals, be indicated by the octanol-water partition coefficient. It can however be affected by many other factors and these will also need to be taken into account.

A9.1.6   It is clearly the objective of a globally harmonized system that, having agreed on a common set of criteria, a common data-set should also be used so that once classified, the classification is globally accepted. For this to occur, there must first be a common understanding of the type of data that can be used in applying the criteria, both in type and quality, and subsequently a common interpretation of the data when measured against the criteria. For that reason, it has been felt necessary to develop a transparent guidance document that would seek to expand and explain the criteria in such a way that a common understanding of their rationale and a common approach to data interpretation may be achieved. This is of particular importance since any harmonized system applied to the "universe of chemicals" will rely heavily on self-classification by manufacturers and suppliers, classifications that must be accepted across national boundaries without always receiving regulatory scrutiny. This guidance document, therefore, seeks to inform the reader, in a number of key areas, and as a result lead to classification in a consistent manner, thus ensuring a truly harmonized and self-operating system.

A9.1.7   Firstly, it will provide a detailed description of the criteria, a rationale for the criteria selected, and an overview of how the scheme will work in practice (Section A9.2). This section will address the common sources of data, the need to apply quality criteria, how to classify when the data-set is incomplete or when a large data-set leads to an ambiguous classification, and other commonly encountered classification problems.

A9.1.8   Secondly, the guidance will provide detailed expert advice on the interpretation of data derived from the available databases, including how to use non-standard data, and specific quality criteria that may apply for individual properties. The problems of data interpretation for "difficult substances", those substances for which standard testing methods either do not apply or give difficult interpretational problems, will be described and advice provided on suitable solutions. The emphasis will be on data interpretation rather than testing since the system will, as far as possible, rely on the best available existing data and data required for regulatory purposes. The three core properties, aquatic toxicity (Section A9.3), degradability (Section A9.4) and bioaccumulation (Section A9.5) are treated separately.

A9.1.9   The range of interpretational problems can be extensive and as a result such interpretation will always rely on the ability and expertise of the individuals responsible for classification. However, it is possible to identify some commonly occurring difficulties and provide guidance that distils accepted expert judgement that can act as an aid to achieving a reliable and consistent result. Such difficulties can fall into a number of overlapping issues:

(a)   The difficulty in applying the current test procedures to a number of types of substance;

(b)   The difficulty in interpreting the data derived both from these "difficult to test" substances and from other substances;

(c)   The difficulty in interpretation of diverse data-sets derived from a wide variety of sources.

A9.1.10   For many organic substances, the testing and interpretation of data present no problems when applying both the relevant OECD Guideline and the classification criteria. There are a number of typical interpretational problems, however, that can be characterized by the type of substance being studied. These are commonly called "difficult substances":

(a)　poorly soluble substances: these substances are difficult to test because they present problems in solution preparation, and in concentration maintenance and verification during aquatic toxicity testing. In addition, many available data for such substances have been produced using "solutions" in excess of the water solubility resulting in major interpretational problems in defining the true $L(E)C_{50}$ for the purposes of classification. Interpretation of the partitioning behaviour can also be problematic where the poor solubility in water and octanol may be compounded by insufficient sensitivity in the analytical method. Water solubility may be difficult to determine and is frequently recorded as simply being less than the detection limit, creating problems in interpreting both aquatic toxicity and bioaccumulation studies. In biodegradation studies, poor solubility may result in low bioavailability and thus lower than expected biodegradation rates. The specific test method or the choice of procedures used can thus be of key importance;

(b)　unstable substances: substance that degrade (or react) rapidly in the test system again present both testing and interpretational problems. It will be necessary to determine whether the correct methodology has been used, whether it is the substance or the degradation/reaction product that has been tested, and whether the data produced is relevant to the classification of the parent substance;

(c)　volatile substances: such substances that can clearly present testing problems when used in open systems should be evaluated to ensure adequate maintenance of exposure concentrations. Loss of test material during biodegradation testing is inevitable in certain methods and will lead to misinterpretation of the results;

(d)　complex or multi-component substances: such substances, for example, hydrocarbon mixtures, frequently cannot be dissolved into a homogeneous solution, and the multiple components make monitoring impossible. Consideration therefore needs to be given to using the data derived from the testing of water accommodated fractions (WAFs) for aquatic toxicity, and the utilization of such data in the classification scheme. Biodegradation, bioaccumulation, partitioning behaviour and water solubility all present problems of interpretation, where each component of the mixture may behave differently;

(e)　polymers: such substances frequently have a wide range of molecular masses, with only a fraction being water soluble. Special methods are available to determine the water soluble fraction and these data will need to be used in interpreting the test data against the classification criteria;

(f)　inorganic compounds and metals: such substances, which can interact with the media, can produce a range of aquatic toxicities dependant on such factors as pH, water hardness etc. Difficult interpretational problems also arise from the testing of essential elements that are beneficial at certain levels. For metals and inorganic metal compounds, the concept of degradability as applied to organic compounds has limited or no meaning. Equally the use of bioaccumulation data should be treated with care;

(g)　surface active substances: such substances can form emulsions in which the bioavailablity is difficult to ascertain, even with careful solution preparation. Micelle formation can result in an overestimation of the bioavailable fraction even when "solutions" are apparently formed. This presents significant problems of interpretation in each of the water solubility, partition coefficient, bioaccumulation and aquatic toxicity studies;

(h)　ionizable substances: such substances can change the extent of ionization according to the level of counter ions in the media. Acids and bases, for example, will show radically different partitioning behaviour depending on the pH;

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(i)   coloured substances: such substance can cause problems in the algal/aquatic plant testing because of the blocking of incident light;

(j)   impurities: some substances can contain impurities that can change in % and in chemical nature between production batches. Interpretational problems can arise where either or both the toxicity and water solubility of the impurities are greater than the parent substance, thus potentially influencing the toxicity data in a significant way.

A9.1.11   These represent some of the problems encountered in establishing the adequacy of data, interpreting the data and applying that data to the classification scheme. Detailed guidance on how to deal with these problems, as well as other issues related will be presented in the following sections. The interpretation of data on aquatic toxicity will be covered in Section A9.3. This section will deal with the specific interpretational problems encountered for the above "difficult substances", including providing some advice on when and how such data can be used within the classification scheme. Also covered will be a general description of the test data used and the testing methodologies suitable for producing such data.

A9.1.12   A wide range of degradation data are available that must be interpreted according to the criteria for rapid degradability. Guidance is thus needed on how to use these data obtained by employing non-standard test methods, including the use of half-lives where these are available, of primary degradation, of soil degradation rates and their suitability for extrapolation to aquatic degradation and of environmental degradation rates. A short description of estimation techniques for evaluating degradability in relation to the classification criteria is also included. This guidance will be provided in Section A9.4.

A9.1.13   Methods by which the potential to bioaccumulate can be determined will be described in Section A9.5. This section will describe the relationship between the partition coefficient criteria and the bioconcentration factor (BCF), provide guidance on the interpretation of existing data, how to estimate the partition coefficient by the use of QSARs when no experimental data are available and in particular deal with the specific problems identified above for difficult substances. The problems encountered when dealing with substances of high molecular mass will also be covered.

A9.1.14   A section is also included which covers general issues concerning the use of QSARs within the system, when and how they may be used, for each of the three properties of concern. As a general approach, it is widely accepted that experimental data should be used rather than QSAR data when such data are available. The use of QSARs will thus be limited to such times when no reliable data are available. Not all substances are suitable for the application of QSAR estimations, however, and the guidance in Section A9.6 will address this issue.

A9.1.15   Finally, a section is devoted to the special problems associated with the classification of metals and their compounds. Clearly, for these compounds, a number of the specific criteria such as biodegradability and octanol-water partition coefficient cannot be applied although the principle of lack of destruction via degradation, and bioaccumulation remain important concepts. Thus it is necessary to adopt a different approach. Metals and metal compounds can undergo interactions with the media which affect the solubility of the metal ion, partitioning from the water column, and the species of metal ion that exists in the water column. In the water column, it is generally the dissolved metal ions which are of concern for toxicity. The interaction of the substance with the media may either increase or decrease the level of ions and hence toxicity. It is thus necessary to consider whether metal ions are likely to be formed from the substance and dissolve in the water, and if so whether they are formed rapidly enough to cause concern. A scheme for interpreting the results from this type of study is presented in Section A9.7.

A9.1.16   While the Guidance Document provides useful advice on how to apply the criteria to a wide variety of situations, it remains a guidance only. It cannot hope to cover all situations that arise in classification. It should therefore be seen as a living document that in part describes the fundamental principles of the system, e.g. hazard based rather than risk based, and the fixed criteria. It must also, in part, be a repository for the accumulated experience in using the scheme to include the interpretations which allow the apparently fixed criteria to be applied in a wide variety of non-standard situations.

A9.2   **The harmonized classification scheme**

A9.2.1   *Scope*

The criteria were developed taking into account existing systems for hazard classification, such as EU- Supply and Use System, the Canadian and US Pesticide systems, GESAMP hazard evaluation procedure, IMO Scheme for Marine Pollutant, the European Road and Rail Transport Scheme (RID/ADR), and the US Land Transport. These systems include supply and subsequent use of chemicals, the sea transport of chemical substances as well as transport of chemical substances by road and rail. The harmonized criteria are therefore intended to identify hazardous chemicals in a common way for use throughout all these systems. To address the needs for all different sectors (transport, supply and use) it was necessary to create two different sub-classes, one Acute sub-class, consisting of three categories and one Chronic sub-class, consisting of 4 categories. The Acute classification sub-class makes provision for two acute hazard categories (Acute 2 and 3) not normally used when considering packaged goods. For substances transported in bulk, there are a number of regulatory decisions that can uniquely arise because of the bulk quantities being considered. For these situations, for example where decisions are required on the ship type to be used, consideration of all acute hazard categories as well as the chronic hazard categories are considered important. The following paragraphs describe in detail the criteria to be used in defining each of these hazard categories.

A9.2.2   *Classification categories and criteria*

The hazard categories for acute and chronic aquatic toxicity and their related criteria are set out in Chapter 4.1, paragraph 4.1.2.2 and Figure 4.1.1.

A9.2.3   *Rationale*

A9.2.3.1   The harmonized system for classification recognizes that the intrinsic hazard to aquatic organisms is represented by both the acute and chronic or longer-term toxicity of a substance, the relative importance of which is determined by the specific regulatory regimes in operation. Distinction can be made between the acute hazard and the chronic hazard and therefore hazard classes are defined for both properties representing a gradation in the level of hazard identified. Clearly the hazard identified by Chronic Category 1 is more severe than Chronic Category 2. Since the acute hazard and chronic hazard represent distinct types of hazard, they are not comparable in terms of their relative severity. Both hazard sub-classes should be applied independently for the classification of substances to establish a basis for all regulatory systems.

A9.2.3.2   The principal hazard classes defined by the criteria relate largely to the potential for chronic hazard. This reflects the overriding concern with respect to chemicals in the environment, namely that the effects caused are usually sub-lethal, e.g. effects on reproduction, and caused by longer-term exposure. While recognizing that the chronic hazard represents the principal concern, particularly for packaged goods where environmental release would be limited in scope, it must also be recognized that chronic toxicity data are expensive to generate and generally not readily available for most substances. On the other hand, acute toxicity data are frequently readily available, or can be generated to highly standardised protocols. It is this acute toxicity which has therefore been used as the core property in defining both the acute and the chronic hazard. Nevertheless, it has been recognized that, where chronic toxicity data are available, it should be possible to use these in defining the appropriate hazard category. The development of specific criteria using such data is thus a high priority in the future development of the scheme.

A9.2.3.3   While recognizing that acute toxicity itself is not a sufficiently accurate predictor of chronic toxicity to be used solely and directly for establishing hazard, it is considered that, in combination with either a potential to bioaccumulate (i.e. a log K_{ow} ≥4 unless BCF <500) or potential longer-term exposure (i.e. lack of rapid degradation) it can be used as a suitable surrogate for classification purposes. Substances that show acute toxicity and also bioaccumulate to a significant degree will normally show chronic toxicity at a significantly lower concentration. Precise acute: chronic ratios are difficult to predict and thus the surrogate data are generally precautionary. Equally substances that do not rapidly degrade have a higher potential for

giving rise to longer term exposures which again may result in long-term toxicity being realized. Thus, for example, Category Chronic 1 should be assigned if either of the following criteria are met:

(a)　　$L(E)C_{50}$ for any appropriate aquatic species ≤ 1 mg/l <u>and</u> a potential to bioaccumulate (log $K_{ow} \geq 4$ unless BCF <500);

(b)　　$L(E)C_{50}$ for any appropriate aquatic species ≤ 1 mg/l <u>and</u> a lack of rapid degradation.

A9.2.3.4　　　The precise definitions of acute toxicity of an appropriate species, lack of rapid degradation and potential to bioaccumulate are detailed in Sections A9.3, A9.4 and A9.5 respectively.

A9.2.3.5　　　For some poorly soluble substances, which are normally considered as those having a water solubility < 1 mg/l, no acute toxicity is expressed in toxicity tests performed at the solubility limit. If for such a substance, however, the BCF ≥ 500, or if absent, the log $K_{ow} \geq 4$ (indicating a bioaccumulating potential) and the substance is also not rapidly degradable, a safety net classification is applied, Chronic Category 4. For these types of substance the exposure duration in short term tests may well be too short for a steady state concentration of the substance to be reached in the test organisms. Thus, even though no acute toxicity has been measured in a short term (acute) test, it remains a real possibility that such non-rapidly degradable and bioaccumulative substances may exert chronic effects, particularly since such low degradability may lead to an extended exposure period in the aquatic environment.

A9.2.3.6　　　In defining acute aquatic toxicity, it is not possible to test all species present in an aquatic ecosystem. Representative species are therefore chosen which cover a range of trophic levels and taxonomic groupings. The taxa chosen, fish, crustacea and aquatic plants that represent the "base-set" in most hazard profiles, represent a minimum data-set for a fully valid description of hazard. The lowest of the available toxicity values will normally be used to define the hazard category. Given the wide range of species in the environment, the three tested can only be a poor surrogate and the lowest value is therefore taken for cautious reasons to define the hazard category. In doing so, it is recognized that the distribution of species sensitivity can be several orders of magnitude wide and that there will thus be both more and less sensitive species in the environment. Thus, when data are limited, the use of the most sensitive species tested gives a cautious but acceptable definition of the hazard. There are some circumstances where it may not be appropriate to use the lowest toxicity value as the basis for classification. This will usually only arise where it is possible to define the sensitivity distribution with more accuracy than would normally be possible, such as when large data-sets are available. Such large data-sets should be evaluated with due caution.

A9.2.4　　　*Application*

A9.2.4.1　　　Generally speaking, in deciding whether a substance should be classified, a search of appropriate databases and other sources of data should be made for the following data elements:

(a)　　water solubility;

(b)　　octanol/water partition coefficient (log K_{ow});

(c)　　fish bioconcentration factor (BCF);

(d)　　acute aquatic toxicity ($L(E)C_{50}$s);

(e)　　chronic aquatic toxicity (NOECs);

(f)　　available degradation (and specifically evidence of ready biodegradability);

(g)　　stability data, in water.

The water solubility and stability data, although not used directly in the criteria, are nevertheless important since they are a valuable help in the data interpretation of the other properties (see A9.1.10).

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A9.2.4.2   To classify, a review should first be made of the available aquatic toxicity data. It will be necessary to consider all the available data and select those which meet the necessary quality criteria for classification. If there are no data available that meet the quality criteria required by the internationally standardized methods, it will be necessary to examine any available data to determine whether a classification can be made. If the data indicate that the acute aquatic toxicity $L(E)C_{50} > 100$ mg/l for soluble substances, then the substance is not classified as hazardous. There are a number of cases where no effects are observed in the test and the aquatic toxicity is thus recorded as a > water solubility value, i.e. there is no acute toxicity within the range of the water solubility in the test media. Where this is the case, and the water solubility in the test media is ≥ 1 mg/l, again, no classification need be applied.

A9.2.4.3   Where the lowest aquatic toxicity data are below 100 mg/l, it is necessary to first decide which hazard category the toxicity falls in, and then to determine whether the chronic and/or the acute sub-class should be applied. This can simply be achieved by examining the available data on the partition coefficient, log K_{ow} and the available data on degradation. If either the log $K_{ow} \geq 4$ or the substance cannot be considered as rapidly degradable, then the appropriate chronic hazard category and the corresponding acute hazard category are applied independently. It should be noted that, although the log K_{ow} is the most readily available indication of a potential to bioaccumulate, an experimentally derived BCF is preferred. Where this is available, this should be used rather than the partition coefficient. In these circumstances, a BCF ≥ 500 would indicate bioaccumulation sufficient to classify in the appropriate chronic hazard class. If the substance is both rapidly degradable and has a low potential to bioaccumulate (BCF < 500 or, if absent log $K_{ow} < 4$) then it should not be assigned to a chronic hazard category, only the acute hazard categories need be applied (see A9.2.1).

A9.2.4.4   For poorly soluble substances, generally speaking, those with a water solubility in the test media of < 1 mg/l, for which no aquatic toxicity has been found, should be further examined to determine whether Chronic Category 4 need be applied. Thus, if the substance is both not rapidly degradable and has a potential to bioaccumulate (BCF ≥ 500 or, if absent log $K_{ow} \geq 4$), the Chronic Category 4 should be applied.

A9.2.5   *Data availability*

The data used to classify a substance can be drawn from data required for regulatory purposes as well as the relevant literature, although a number of internationally recognized data-bases exist which can act as a good starting point. Such databases vary widely in quality and comprehensiveness and it is unlikely that any one database will hold all he information necessary for classification to be made. Some databases specialize in aquatic toxicity and others in environmental fate. There is an obligation on the chemical supplier to make the necessary searches and checks to determine the extent and quality of the data available and to use it in assigning the appropriate hazard category.

A9.2.6   *Data quality*

A9.2.6.1   The precise use of the available data will be described in the relevant section but, as a general rule, data generated to standard international guidelines and to GLP is to be preferred over other types of data. Equally, however, it is important to appreciate that classification can be made based on the best available data. Thus if no data is available which conforms to the quality standard detailed above, classification can still be made provided the data used is not considered invalid. To assist this process, a quality scoring guide has been developed and used extensively in a number of fora and generally conforms to the following categories:

(a)   Data derived from official data sources that have been validated by regulatoryauthorities, such as EU Water Quality Monographs, US-EPA Water Quality Criteria. These data can be considered as valid for classification purposes. No assumption should be made that these are the only data available, however, and due regard should be given to the date of the relevant report. Newly available data may not have been considered;

(b)   Data derived from recognized international guidelines (e.g. OECD Guidelines) or national guidelines of equivalent quality. Subject to the data interpretation issues raised in the following sections, these data can be used for classification;

(c)   Data derived from testing which, while not strictly according to a guideline detailed above, follows accepted scientific principles and procedures and/or has been peer reviewed prior to publication. For such data, where all the experimental detail is not recorded, some judgement may be required to determine validity. Normally, such data may be used within the classification scheme;

(d)   Data derived from testing procedures which deviate significantly from standard guidelines and are considered as unreliable, should not be used in classification;

(e)   QSAR data. The circumstances of use and validity of QSAR data are discussed in the relevant sections;

(f)   Data derived from secondary sources such as handbooks, reviews, citation, etc. where the data quality cannot be directly evaluated. Such data should be examined where data from quality 1, 2 and 3 are not available, to determine whether it can be used. Such data should have sufficient detail to allow quality to be assessed. In determining the acceptability of these data for the purposes of classification, due regard should be given to the difficulties in testing that may have affected data quality and the significance of the reported result in terms of the level of hazard identified (see A9.3.6.2.3).

A9.2.6.2   Classification may also be made on incomplete toxicity data-sets, e.g. where data are not available on all three trophic levels. In these cases, the classification may be considered as "provisional" and subject to further information becoming available. In general, all the data available will need to be considered prior to assigning a classification. Where good quality data are not available, lower quality data will need to be considered. In these circumstances, a judgement will need to be made regarding the true level of hazard. For example, where good quality data are available for a particular species or taxa, this should be used in preference to any lower quality data which might also be available for that species or taxa. However, good quality data may not always be available for all the basic data set trophic levels. It will be necessary to consider data of lower quality for those trophic levels for which good quality data are not available. Consideration of such data, however, will also need to consider the difficulties that may have affected the likelihood of achieving a valid result. For example, the test details and experimental design may be critical to the assessment of the usability of some data, such as that from hydrolytically unstable chemicals, while less so for other chemicals. Such difficulties are described further in Section A9.3.

A9.2.6.3   Normally, the identification of hazard, and hence the classification will be based on information directly obtained from testing of the substance being considered. There are occasions, however, where this can create difficulties in the testing or the outcomes do not conform to common sense. For example, some chemicals, although stable in the bottle, will react rapidly (or slowly) in water giving rise to degradation products that may have different properties. Where such degradation is rapid, the available test data will frequently define the hazard of the degradation products since it will be these that have been tested. These data may be used to classify the parent substance in the normal way. However, where degradation is slower, it may be possible to test the parent substance and thus generate hazard data in the normal manner. The subsequent degradation may then be considered in determining whether an acute or chronic hazard class should apply. There may be occasions, however, when a substance so tested may degrade to give rise to a more hazardous product. In these circumstances, the classification of the parent should take due account of the hazard of the degradation product, and the rate at which it can be formed under normal environmental conditions.

A9.3   **Aquatic toxicity**

A9.3.1   *Introduction*

The basis for the identification of hazard to the aquatic environment for a substance is the aquatic toxicity of that substance. Classification is predicated on having toxicity data for fish, crustacea, and algae/aquatic plant available. These taxa are generally accepted as representative of aquatic fauna and flora for hazard identification. Data on these particular taxa are more likely to be found because of this general acceptance by regulatory authorities and the chemical industry. Other information on the degradation and bioaccumulation behaviour is used to better delineate the aquatic hazard. This section describes the appropriate tests for ecotoxicity, provides some basic concepts in evaluating the data and using combinations of testing results for classification, summarizes approaches for dealing with difficulty substances, and includes a brief discussion on interpretation of data quality.

A9.3.2   *Description of tests*

A9.3.2.1   For classifying substances in the harmonized system, freshwater and marine species toxicity data can be considered as equivalent data. It should be noted that some types of substances, e.g. ionizable organic chemicals or organometallic substances may express different toxicities in freshwater and marine environments. Since the purpose of classification is to characterize hazard in the aquatic environment, the result showing the highest toxicity should be chosen.

A9.3.2.2   The GHS criteria for determining health and environmental hazards should be test method neutral, allowing different approaches as long as they are scientifically sound and validated according to international procedures and criteria already referred to in existing systems for the endpoints of concern and produce mutually acceptable data. According to the proposed system (OECD 1998):

> *"Acute toxicity would normally be determined using a fish 96 hour LC50 (OECD Test Guideline 203 or equivalent), a crustacea species 48 hour EC50 (OECD Test Guideline 202 or equivalent) and/or an algal species 72 or 96 hour EC50 (OECD Test Guideline 201 or equivalent). These species are considered as surrogate for all aquatic organisms and data on other species such as the duckweed Lemna may also be considered if the test methodology is suitable."*

Chronic testing involves an exposure that is lingering or continues for a longer time; the term can signify periods from days to a year, or more depending on the reproductive cycle of the aquatic organism. Chronic tests can be done to assess certain endpoints relating to growth, survival, reproduction and development.

> *"Chronic toxicity data are less available than acute data and the range of testing procedures less standardised. Data generated according to the OECD Test Guidelines 210 (Fish Early Life Stage), 202 Part 2 or 211 (Daphnia Reproduction) and 201 (Algal Growth Inhibition) can be accepted. Other validated and internationally accepted tests could also be used. The NOECs or other equivalent L(E)Cx should be used."*

A9.3.2.3   It should be noted that several of the OECD guidelines cited as examples for classification are being revised or are being planned for updating. Such revisions may lead to minor modifications of test conditions. Therefore, the expert group that developed the harmonized criteria for classification intended some flexibility in test duration or even species used.

A9.3.2.4   Guidelines for conducting acceptable tests with fish, crustacea, and algae can be found in many sources (OECD, 1999; EPA, 1996; ASTM, 1999; ISO EU). The OECD monograph No.11, Detailed Review Paper on Aquatic Toxicity Testing for Industrial Chemicals and Pesticides, is a good compilation of pelagic test methods and sources of testing guidance. This document is also a source of appropriate test methodologies.

A9.3.2.5   *Fish Tests*

A9.3.2.5.1   Acute testing

Acute tests are generally performed with young juveniles 0.1 - 5 g in size for a period of 96 hours. The observational endpoint in these tests is mortality. Fish larger than this range and/or durations shorter than 96 hours are generally less sensitive. However, for classification, they could be used if no acceptable data with the smaller fish for 96 hours are available or the results of these tests with different size fish or test durations would influence classification in a more hazardous category. Tests consistent with OECD Test Guideline 203 (Fish 96 hour LC50) or equivalent should be used for classification.

A9.3.2.5.2   Chronic testing

Chronic or long term tests with fish can be initiated with fertilized eggs, embryos, juveniles, or reproductively active adults. Tests consistent with OECD Test Guideline 210 (Fish Early Life Stage), the fish life-cycle test (US EPA 850.1500), or equivalent can be used in the classification scheme. Durations can vary widely depending on the test purpose (anywhere from 7 days to over 200 days). Observational endpoints can include hatching success, growth (length and weight changes), spawning success, and survival. Technically, the OECD 210 Guideline (Fish Early Life Stage) is not a "chronic" test, but a sub-chronic test on sensitive life stages. It is widely accepted as a predictor of chronic toxicity and is used as such for purposes of classification in the harmonized system. Fish early life stage toxicity data are much more available than fish life cycle or reproduction studies.

A9.3.2.6   *Crustacea Tests*

A9.3.2.6.1   Acute testing

Acute tests with crustacea generally begin with first instar juveniles. For daphnids, a test duration of 48 hours is used. For other crustacea, such as mysids or others, a duration of 96 hours is typical. The observational endpoint is mortality or immobilization as a surrogate to mortality. Immobilization is defined as unresponsive to gentle prodding. Tests consistent with OECD Test Guideline 202 Part 1 (Daphnia acute) or USA-EPA OPPTS 850.1035 (Mysid acute toxicity) or their equivalents should be used for classification.

A9.3.2.6.2   Chronic testing

Chronic tests with crustacea also generally begin with first instar juveniles and continue through maturation and reproduction. For daphnids, 21 days is sufficient for maturation and the production of 3 broods. For mysids, 28 days is necessary. Observational endpoints include time to first brood, number of offspring produced per female, growth, and survival. It is recommended that tests consistent with OECD Test Guideline 202 Part 2 (Daphnia reproduction) or US-EPA 850.1350 (Mysid chronic) or their equivalents be used in the classification scheme.

A9.3.2.7   *Algae/Plant Tests*

A9.3.2.7.1   Tests in algae

Algae are cultured and exposed to the test substance in a nutrient-enriched medium. Tests consistent with OECD Test Guideline 201 (Algal growth inhibition) should be used. Standard test methods employ a cell density in the inoculum in order to ensure exponential growth through the test, usually 3 to 4 days duration.

The algal test is a short-term test and, although it provides both acute and chronic endpoints, only the acute EC_{50} is used for classification in the harmonized system. The preferred observational endpoint in this study is algal growth rate inhibition because it is not dependent on the test design, whereas biomass depends both on growth rate of the test species as well as test duration and other elements of test design. If the endpoint is

reported only as reduction in biomass or is not specified, then this value may be interpreted as an equivalent endpoint.

A9.3.2.7.2   Tests in aquatic macrophytes

The most commonly used vascular plants for aquatic toxicity tests are duckweeds (*Lemna gibba* and *Lemna minor*). The Lemna test is a short-term test and, although it provides both acute and sub-chronic endpoints, only the acute EC_{50} is used for classification in the harmonized system. The tests last for up to 14 days and are performed in nutrient enriched media similar to that used for algae, but may be increased in strength. The observational endpoint is based on change in the number of fronds produced. Tests consistent with OECD Test Guideline on Lemna (in preparation) and US-EPA 850.4400 (aquatic plant toxicity, Lemna) should be used.

A9.3.3   *Aquatic toxicity concepts*

This section addresses the use of acute and chronic toxicity data in classification, and special considerations for exposure regimes, algal toxicity testing, and use of QSARs. For a more detailed discussion of aquatic toxicity concepts, one can refer to Rand (1996).

A9.3.3.1   *Acute toxicity*

A9.3.3.1.1   Acute toxicity for purposes of classification refers to the intrinsic property of a substance to be injurious to an organism in a short-term exposure to that substance. Acute toxicity is generally expressed in terms of a concentration which is lethal to 50% of the test organisms (LC_{50}), causes a measurable adverse effect to 50% of the test organisms (e.g. immobilization of daphnids), or leads to a 50% reduction in test (treated) organism responses from control (untreated) organism responses (e.g. growth rate in algae).

A9.3.3.1.2   Substances with an acute toxicity determined to be less than one part per million (1 mg/l) are generally recognized as being very toxic. The handling, use, or discharge into the environment of these substances poses a high degree of hazard and they are classified in Chronic and/or Acute Category 1. Decimal bands are accepted for categorizing acute toxicity above this category. Substances with an acute toxicity measured from one to ten parts per million (1 - 10 mg/l) are classified in Category 2 for acute toxicity, from ten to one hundred parts per million (10 - 100 mg/l) are classified in Category 3 for acute toxicity, and those over one hundred parts per million (> 100 mg/l) are regarded as practically non-toxic.

A9.3.3.2   *Chronic toxicity*

A9.3.3.2.1   Chronic toxicity, for purposes of classification, refers to the potential or actual properties of a substance to cause adverse effects to aquatic organisms during exposures which are determined in relation to the life-cycle of the organism. Such chronic effects usually include a range of sublethal endpoints and are generally expressed in terms of a No Observable Effect Concentration (NOEC), or an equivalent ECx. Observable endpoints typically include survival, growth and/or reproduction. Chronic toxicity exposure durations can vary widely depending on test endpoint measured and test species used.

A9.3.3.2.2   Since chronic toxicity data are less common in certain sectors than acute data, for classification schemes, the potential for chronic toxicity is identified by appropriate combinations of acute toxicity, lack of degradability, and/or the potential or actual bioaccumulation. Where such data exist and show long-term NOECs > 1 mg/l, this can be taken into account when deciding whether the classification based on the acute data should be applied. In this context, the following general approach should be used. In order to remove a chronic classification, it must be demonstrated that the NOEC used would be suitable in removing the concern for all taxa which resulted in classification. This can often be achieved by showing a long-term NOEC >1 mg/l for the most sensitive species identified by the acute toxicity. Thus, if a classification has been applied based on a fish acute LC50, it would generally not be possible to remove this classification using a long-term NOEC from an invertebrate toxicity test. In this case, the NOEC would normally need to be derived from a long-term fish test of the same species or one of equivalent or greater sensitivity. Equally, if classification has resulted from the acute toxicity to more than one taxa, it is likely that NOECs > 1 mg/l from each taxa will need to be demonstrated.

In case of classification of a substance as Chronic Category 4, it is sufficient to demonstrate that NOECs are greater than the water solubility of the substances under consideration.

A9.3.3.2.3   Testing with algae/Lemna cannot be used for de-classifying chemicals because (1) the algae and Lemna tests are not long-term studies, (2) the acute to chronic ratio is generally narrow and (3) the endpoints are more consistent with the end points for other organisms.

However where classification is applied solely due to the acute toxicity (L(E)C$_{50}$) observed in single algae/aquatic plant tests, but there is evidence from a range of other algae tests that the chronic toxicity (NOECs) for this taxonomic group is above 1mg/l, this evidence could be used to consider declassification. At present this approach cannot be applied to aquatic plants since no standardized chronic toxicity tests have been developed.

A9.3.3.2.4   The GHS is intended to contain a specific value of chronic toxicity below which substances would be classified as chronically toxic, but the criteria are not yet set.

A9.3.3.3   *Exposure regimes*

Four types of exposure conditions are employed in both acute and chronic tests and in both freshwater and saltwater media: static, static-renewal (semi-static), recirculation, and flow-through. The choice for which test type to use usually depends on test substance characteristics, test duration, test species, and regulatory requirements.

A9.3.3.4   *Test media for algae*

Algal tests are performed in nutrient-enriched media and use of one common constituent, EDTA, or other chelators, should be considered carefully. When testing the toxicity of organic chemicals, trace amounts of a chelator like EDTA are needed to complex micronutrients in the culture medium; if omitted, algal growth can be significantly reduced and compromise test utility. However, chelators can reduce the observed toxicity of metal test substances. Therefore, for metal compounds, it is desirable that data from tests with high concentration of chelators and/or tests with stoichiometrical excess of chelator relative to iron be critically evaluated. Free chelator may mask heavy metal toxicity considerably, in particular with strong chelators like EDTA. However, in the absence of available iron in the medium the growth of algae can become iron limited, and consequently data from tests with no or with reduced iron and EDTA should be treated with caution.

A9.3.3.5   *Use of QSARs*

For purpose of classification, and in the absence of experimental data, QSARs can be relied upon to provide predictions of acute toxicity for fish, daphnia, and algae for non-electrolyte, non-electrophilic, and otherwise non-reactive substances (See Section A9.6 on *Use of QSAR*). Problems remain for substances such as organophosphates which operate by means of special mechanisms such as functional groups which interact with biological receptors, or which can form sulfhydryl bonds with cellular proteins. Reliable QSARs have been derived for chemicals acting by a basic narcosis mechanism. These chemicals are nonelectrolytes of low reactivity such as hydrocarbons, alcohols, ketones and certain aliphatic chlorinated hydrocarbons which produce their biological effects as a function of their partition coefficients. Every organic chemical can produce narcosis. However, if the chemical is an electrolyte or contains specific functional groups leading to non-narcotic mechanisms as well, any calculations of toxicity based on partition coefficient alone would severely underestimate the toxicity. QSARs for acute aquatic toxicity of parent compounds cannot be used to predict the effects of toxic metabolites or degradates, when these arise after a longer time period than the duration of acute tests.

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A9.3.4   *Weight of evidence*

A9.3.4.1   The best quality data should be used as the fundamental basis for classification. Classification should preferably be based on primary data sources. It is essential that test conditions be clearly and completely articulated.

A9.3.4.2   Where multiple studies for a taxonomic group are available, a decision on what is the most sensitive and highest quality must be made. A judgement has to be made on a case by case basis whether a non-GLP study with a more sensitive observation is used in lieu of a GLP study. It would appear that results that indicate high toxicity from tests performed according to non-standard or non-GLP guidelines should be able to be used for classification, whereas studies, which demonstrate negligible toxicity, would require more careful consideration. Substances, which are difficult to test, may yield apparent results that are more or less severe than the true toxicity. Expert judgement would also be needed for classification in these cases.

A9.3.4.3   Where more than one acceptable test is available for the same taxonomic group, the most sensitive (the one with the lowest L(E)C50 or NOEC) is generally used for classification. However, this must be dealt with on a case-by-case basis. When larger data sets (4 or more values) are available for the same species, the geometric mean of toxicity values may be used as the representative toxicity value for that species. In estimating a mean value, it is not advisable to combine tests of different species within a taxa group or in different life stages or tested under different conditions or duration.

A9.3.5   *Difficult to test substances*

A9.3.5.1   Valid aquatic toxicity tests require the dissolution of the test substance in the water media under the test conditions recommended by the guideline. In addition, a bioavailable exposure concentration should be maintained for the duration of the test. Some chemical substances are difficult to test in aquatic systems and guidance has been developed to assist in testing these materials (DoE 1996; ECETOC 1996; and US EPA 1996). OECD is in the process of finalizing a Guidance Document on Aquatic Toxicity testing of Difficult Substances and Mixtures (OECD, 2000). This latter document is a good source of information on the types of substances that are difficult to test and the steps needed to ensure valid conclusions from tests with these materials.

A9.3.5.2   Nevertheless, much test data exist that may have used testing methodologies which, while not in conformity with what might be considered best practice today, can still yield information suitable for application of the classification criteria. Such data require special guidance on interpretation, although ultimately, expert judgement must be used in determining data validity. Such difficult to test substances may be poorly soluble, volatile, or subject to rapid degradation due to such processes as phototransformation, hydrolysis, oxidation, or biotic degradation. When testing algae, coloured materials may interfere with the test endpoint by attenuating the light needed for cell growth. In a similar manner, substances tested as cloudy dispersions above solubility may give rise to false toxicity measurements. Loading of the water column with test material can be an issue for particulates or solids such as metals. Petroleum distillate fractions can also pose loading problems, as well as difficult interpretational problems when deciding on the appropriate concentrations for determining $L(E)C_{50}$ values. The draft Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures describes the more common properties of many types of substances which are likely to pose testing difficulties.

        (a)   <u>Stability:</u> If test chemical concentrations are expected to fall below 80% of nominal, testing, in order to be valid, may require exposure regimes which provide for renewal of the test material. Semi-static or flow-through conditions are preferred. Special problems arise, therefore, with respect to testing on algae, where the standard guidelines generally include static tests to be conducted. While alternative exposure regimes are possible for crustacea and fish, these tests are frequently conducted on static conditions as included in the internationally agreed guidelines. In these tests, a certain level of degradation as well as other relevant factors has to be tolerated and appropriate account must be taken in calculations of toxic concentrations. Some approaches on how this can be dealt with are covered in A9.3.5.6. Where degradation occurs, it is also important to consider the influence of the toxicity of the degradation products on the recorded toxicity in the test.

Expert judgement will need to be exercised when deciding if the data can be used for classification;

(b) Degradation: When a compound breaks down or degrades under test condition, expert judgement should be used in calculating toxicity for classification, including consideration of known or likely breakdown products. Concentrations of the parent material and all significant toxic degradates are desirable. If degradates are expected to be relatively non-toxic, renewable exposure regimes are desirable in order to ensure that levels of the parent compounds are maintained;

(c) Saturation: For single component substances, classification should be based only on toxic responses observed in the soluble range, and not on total chemical loading above solubility. Frequently, data are available which indicate toxicity at levels in excess of water solubility and, while these data will often be regarded as not valid, some interpretation may be possible. These problems generally apply when testing poorly soluble substances, and guidance on how to interpret such data is included in A9.3.5.7 (see also the Guidance document on aquatic toxicity testing of difficult substances and mixtures);

(d) Perturbation of test media: Special provisions may be needed to ensure dissolution of difficult to test substances. Such measures should not lead to significant changes in the test media when such changes are likely to lead to an increase or decrease in the apparent toxicity and hence the classification level of the test substance;

(e) Complex substances: Many substances covered by the classification scheme are in fact mixtures, for which measurement of exposure concentrations is difficult, and in some cases impossible. Substances such as petroleum distillate fractions, polymers, substances with significant levels of impurities, etc can pose special problems since the toxic concentration is difficult to define and impossible to verify. Typical testing procedures often rely on the formation of a Water Soluble Fraction (WSF) or Water Accommodated Fraction (WAF) and data are reported in terms of loading rates. These data may be used in applying the classification criteria.

A9.3.5.3   For classification of organic compounds, it is desirable to have stabilized and analytically measured test concentrations. Although measured concentrations are preferred, classification may be based on nominal concentration studies when these are the only valid data available under certain circumstances. If the material is likely to substantially degrade or otherwise be lost from the water column, care must be taken in data interpretation and classification should be done taking the loss of the toxicant during the test into account, if relevant and possible. Additionally, metals present their own set of difficulties and are discussed separately. Table A9.3.1 lists several properties of difficult to test substances and their relevance for classification.

A9.3.5.4   In most difficult to test conditions, the actual test concentration is likely to be less than the nominal or expected test concentration. Where toxicities ($L(E)C_{50}s$) are estimated to be < 1 mg/l for a difficult to test substance, one can be fairly confident the classification in the Acute Category 1 (and Chronic Category 1 if appropriate) is warranted. However, if the estimated toxicity is > 1 mg/l, the estimated toxicity is likely to under-represent the toxicity. In these circumstances, expert judgement is needed to determine the acceptability of a test with a difficult to test substance for use in classification. Where the nature of the testing difficulty is believed to have a significant influence on the actual test concentration when toxicity is estimated to be > 1 mg/l and the test concentration is not measured, then the test should be used with due caution in classification.

A9.3.5.5   The following paragraphs provide some detailed guidance on some of these interpretational problems. In doing so it should be remembered that this is guidance and hard and fast rules cannot be applied. The nature of many of the difficulties mean that expert judgement must always be applied both in determining whether there is sufficient information in a test for a judgement to be made on its validity, and also whether a toxicity level can be determined suitable for use in applying the classification criteria.

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A9.3.5.6   *Unstable substances*

A9.3.5.6.1   While testing procedures should ideally have been adopted which minimized the impacts of instability in the test media, in practice, in certain tests, it can be almost impossible to maintain a concentration throughout the test. Common causes of such instability are oxidation, hydrolysis, photodegradation and biodegradation. While the latter forms of degradation can more readily be controlled, such controls are frequently absent in much existing testing. Nevertheless, for some testing, particularly acute and chronic fish toxicity testing, a choice of exposure regimes is available to help minimize losses due to instability, and this should be taken into account in deciding on the test data validity.

A9.3.5.6.2   Where instability is a factor in determining the level of exposure during the test, an essential prerequisite for data interpretation is the existence of measured exposure concentrations at suitable time points throughout the test. In the absence of analytically measured concentrations at least at the start and end of test, no valid interpretation can be made and the test should be considered as invalid for classification purposes. Where measured data are available, a number of practical rules can be considered by way of guidance in interpretation:

(a)   where measured data are available for the start and end of test (as is normal for the acute Daphnia and algal tests), the $L(E)C_{50}$, for classification purposes, may be calculated based on the geometric mean of the start and end of test concentrations. Where the end of test concentrations are below the analytical detection limit, such concentrations shall be considered to be half that detection limit;

(b)   where measured data are available at the start and end of media renewal periods (as may be available for the semi-static tests), the geometric mean for each renewal period should be calculated, and the mean exposure over the whole exposure period calculated from these data;

(c)   where the toxicity can be attributed to a degradation breakdown product, and the concentrations of this are known, the $L(E)C_{50}$ for classification purposes, may be calculated based on the geometric mean of the degradation product concentration, back calculated to the parent substance;

(d)   similar principles may be applied to measured data in chronic toxicity testing.

A9.3.5.7   *Poorly soluble substances*

A9.3.5.7.1   These substances, usually taken to be those with a solubility in water <1 mg/l, are frequently difficult to dissolve in the test media, and the dissolved concentrations will often prove difficult to measure at the low concentrations anticipated. For many substances, the true solubility in the test media will be unknown, and will often be recorded as < detection limit in purified water. Nevertheless such substances can show toxicity, and where no toxicity is found, judgement must be applied to whether the result can be considered valid for classification. Judgement should err on the side of caution and should not underestimate the hazard.

A9.3.5.7.2   Ideally, tests using appropriate dissolution techniques and with accurately measured concentrations within the range of water solubility should be used. Where such test data are available, they should be used in preference to other data. It is normal, however, particularly when considering older data, to find such substances with toxicity levels recorded in excess of the water solubility, or where the dissolved levels are below the detection limit of the analytical method. Thus, in both circumstances, it is not possible to verify the actual exposure concentrations using measured data. Where these are the only data available on which to classify, some practical rules can be considered by way of general guidance:

(a)   where the acute toxicity is recorded at levels in excess of the water solubility, the $L(E)C_{50}$ for classification purposes, may be considered to be equal to or below the measured water solubility. In such circumstances it is likely that Chronic Category 1 and/or Acute Category 1 should be applied. In making this decision, due attention should be paid to the possibility that the excess undissolved substance may have given rise to physical effects

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on the test organisms. Where this is considered the likely cause of the effects observed, the test should be considered as invalid for classification purposes;

(b)   where no acute toxicity is recorded at levels in excess of the water solubility, the $L(E)C_{50}$ for classification purposes may be considered to be greater than the measured water solubility. In such circumstances, consideration should be given to whether the Chronic Category 4 should apply. In making a decision that the substance shows no acute toxicity, due account should be taken of the techniques used to achieve the maximum dissolved concentrations. Where these are not considered as adequate, the test should be considered as invalid for classification purposes;

(c)   where the water solubility is below the detection limit of the analytical method for a substance, and acute toxicity is recorded, the $L(E)C_{50}$ for classification purposes, may be considered to be less than the analytical detection limit. Where no toxicity is observed, the $L(E)C_{50}$ for classification purposes, may be considered to be greater than the water solubility. Due consideration should also be given to the quality criteria mentioned above;

(d)   where chronic toxicity data are available, the same general rules should apply. In principle, only data showing no effects at the water solubility limit, or greater than 1 mg/l need be considered. Again, where these data cannot be validated by consideration of measured concentrations, the techniques used to achieve the maximum dissolved concentrations must be considered as appropriate.

A9.3.5.8   *Other factors contributing to concentration loss*

        A number of other factors can also contribute to losses of concentration and, while some can be avoided by correct study design, interpretation of data where these factors have contributed may, from time to time, be necessary.

(a)   sedimentation: this can occur during a test for a number of reasons. A common explanation is that the substance has not truly dissolved despite the apparent absence of particulates, and agglomeration occurs during the test leading to precipitation. In these circumstances, the $L(E)C_{50}$ for classification purposes, may be considered to be based on the end of test concentrations. Equally, precipitation can occur through reaction with the media. This is considered under instability above;

(b)   adsorption: this can occur for substances of high adsorption characteristics such as high log Kow substances. Where this occurs, the loss of concentration is usually rapid and exposure may best be characterized by the end of test concentrations;

(c)   bioaccumulation: losses may occur through the bioaccumulation of a substance into the test organisms. This may be particularly important where the water solubility is low and log K_{ow} correspondingly high. The $L(E)C_{50}$ for classification purposes, may be calculated based on the geometric mean of the start and end of test concentrations.

A9.3.5.9   *Perturbation of the test media*

A9.3.5.9.1   Strong acids and bases may appear toxic because they may alter pH. Generally however changes of the pH in aquatic systems are normally prevented by buffer systems in the test medium. If no data are available on a salt, the salt should generally be classified in the same way as the anion or cation, i.e. as the ion that receives the most stringent classification. If the effect concentration is related to only one of the ions, the classification of the salt should take the molecular weight difference into consideration by correcting the effect concentration by multiplying with the ratio: $MW_{salt}/MW_{ion.}$

A9.3.5.9.2   Polymers are typically not available in aquatic systems. Dispersible polymers and other high molecular mass materials can perturb the test system and interfere with uptake of oxygen, and give rise to mechanical or secondary effects. These factors need to be taken into account when considering data from these substances. Many polymers behave like complex substances, however, having a significant low molecular mass fraction which can leach from the bulk polymer. This is considered further below.

A9.3.5.10   *Complex substances*

A9.3.5.10.1   Complex substances are characterized by a range of chemical structures, frequently in a homologous series, but covering a wide range of water solubilities and other physico-chemical characteristics. On addition to water, an equilibrium will be reached between the dissolved and undissolved fractions which will be characteristic of the loading of the substance. For this reason, such complex substances are usually tested as a WSF or WAF, and the $L(E)C_{50}$ recorded based on the loading or nominal concentrations. Analytical support data are not normally available since the dissolved fraction will itself be a complex mixtures of components. The toxicity parameter is sometimes referred to as LL_{50}, related to the lethal loading level. This loading level from the WSF or WAF may be used directly in the classification criteria.

A9.3.5.10.2   Polymers represent a special kind of complex substance, requiring consideration of the polymer type and their dissolution/dispersal behaviour. Polymers may dissolve as such without change, (true solubility related to particle size), be dispersible, or portions consisting of low molecular weight fractions may go into solution. In the latter case, in effect, the testing of a polymer is a test of the ability of low molecular mass material to leach from the bulk polymer, and whether this leachate is toxic. It can thus be considered in the same way as a complex mixture in that a loading of polymer can best characterize the resultant leachate, and hence the toxicity can be related to this loading.

Table A9.3.1   Classification of difficult test substances

Property	Nature of difficulty	Relevance for classification
Poorly water soluble	Achieving/maintaining required exposure concentration. Analysing exposure.	When toxic responses are observed above apparent solubility, expert judgement is required to confirm whether effects are due to chemical toxicity or a physical effect; if no effects are observed, it should be demonstrated that full, saturated dissolution has been achieved.
Toxic at low concentrations	Achieving/maintaining required exposure concentration. Analysing exposure.	Classified based on toxicity < 1 mg/l
Volatile	Maintaining and measuring exposure concentration.	Classification should be based on reliable measurement of concentrations.
Photo-degradable	Maintaining exposure concentrations. Toxicity of breakdown products.	Classification requires expert judgement and should be based on measured concentrations. Toxicity of significant breakdown products should be characterized.
Hydrolytically unstable	Maintaining exposure concentrations. Toxicity of breakdown products. Comparison of degradation half-lives to the exposure regimen used in testing.	Classification requires expert judgement, should be based on measured concentrations, and needs to address the toxicity of significant breakdown products.
Oxidizable	Achieving, maintaining and measuring exposure concentration. Toxicity of modified chemical structures or breakdown products. Comparison of degradation half-lives to the exposure regimen used in testing.	Classification requires expert judgement, should be based on measured concentrations, and needs to address the toxicity of significant breakdown products.
Subject to corrosion/ transformation (this refers to metals /metal compounds)	Achieving, maintaining and measuring exposure concentration. Comparison of partitioning from the water column half-lives to the exposure regimen used in testing.	Classification requires expert judgement, should be based on measured concentrations, and needs to address the toxicity of significant breakdown products.

Property	Nature of difficulty	Relevance for classification
Biodegradable	Maintaining exposure concentrations. Toxicity of breakdown products. Comparison of degradation half-lives to the exposure regimen used in testing.	Classification requires expert judgement, should be based on measured concentrations, and needs to address the toxicity of significant breakdown products.
Adsorbing	Maintaining exposure concentrations. Analysing exposure. Toxicity mitigation due to reduced availability of test substance.	Classification should use measured concentration of available material.
Chelating	Distinguishing chelated and non-chelated fractions in media.	Classification should use measurement of concentration of bioavailable material.
Coloured	Light attenuation (an algal problem).	Classification must distinguish toxic effects from reduced growth due to light attenuation.
Hydrophobic	Maintaining constant exposure concentrations.	Classification should use measured concentration.
Ionized	Maintaining exposure concentrations. Toxicity of breakdown products. Comparison of degradation half-lives to the exposure regime used in testing.	Classification requires expert judgement, should be based on measured concentrations, and needs to address the toxicity of significant breakdown products.
Multi-component	Preparing representative test batches.	Considered same as complex mixture.

A9.3.6 *Interpreting data quality*

A9.3.6.1 *Standardization*

Many factors can influence the results of toxicity tests with aquatic organisms. These factors include characteristics of the test water, experimental design, chemical characteristics of the test material, and biological characteristics of the test organisms. Therefore, it is important in conducting aquatic toxicity tests to use standardized test procedures to reduce the influence of these sources of extraneous variability. The goal of test standardization and international harmonization of these standards is to reduce test variability and improve precision, reproducibility, and consistency of test results.

A9.3.6.2 *Data hierarchies*

A9.3.6.2.1 Classification should be based on primary data of good quality. Preference is given to data conforming to OECD Test Guidelines or equivalent and Good Laboratory Practices (GLP). While data from internationally harmonized test methods performed on standard test species are preferred, results of tests performed using widely recognized international or national methods or their equivalent may also be used, e.g. ISO or ASTM methods. Data from tests that appear to conform to accepted guidelines but which lacks provisions for GLP can be used in the absence of pertinent GLP data.

A9.3.6.2.2 Pedersen et al (1995) provides a data quality-scoring system, which is compatible with many others in current use, including that, used by the US-EPA for its AQUIRE database. See also Mensink et al (1995) for discussions of data quality. The data quality scoring system described in Pedersen *et al*. includes a reliability ranking scheme, which can be a model for use with in classifying under the harmonized scheme. The first three levels of data described by Pedersen are for preferred data.

A9.3.6.2.3 Data for classification under the harmonized scheme should come from primary sources. However, since many nations and regulatory authorities will perform classification using the globally harmonized scheme, classification should allow for use of reviews from national authorities and expert panels as long as the reviews are based on primary sources. Such reviews should include summaries of test conditions, which are sufficiently detailed for weight of evidence and classification decisions to be made. It may be possible to use the reviews, which were made by a well-recognized group such as GESAMP for which the primary data are accessible.

A9.3.6.2.4 In the absence of empirical test data, validated Quantitative Structure Activity Relationships (QSARs) for aquatic toxicity may be used. Test data always take precedence over QSAR predictions, providing the test data are valid.

A9.4 **Degradation**

A9.4.1 *Introduction*

A9.4.1.1   Degradability is one of the important intrinsic properties of chemical substances that determine their potential environmental hazard. Non-degradable substances will persist in the environment and may consequently have a potential for causing long-term adverse effects on biota. In contrast, degradable substances may be removed in the sewers, in sewage treatment plants or in the environment.

Classification of chemical substances is primarily based on their intrinsic properties. However, the degree of degradation depends not only on the intrinsic recalcitrance of the molecule, but also on the actual conditions in the receiving environmental compartment as e.g. redox potential, pH, presence of suitable micro-organisms, concentration of the substances and occurrence and concentration of other substrates. The interpretation of the degradation properties in an aquatic hazard classification context therefore requires detailed criteria that balance the intrinsic properties of the substance and the prevailing environmental conditions into a concluding statement on the potential for long-term adverse effects. The purpose of the present section is to present guidance for interpretation of data on degradability of organic substances. The guidance is based on an analysis of the above mentioned aspects regarding degradation in the aquatic environment. Based on the guidance a detailed decision scheme for use of existing degradation data for classification purposes is proposed. The types of degradation data included in this Guidance Document are ready biodegradability data, simulation data for transformation in water, aquatic sediment and soil, BOD_5/COD-data and techniques for estimation of rapid degradability in the aquatic environment. Also considered are anaerobic degradability, inherent biodegradability, sewage treatment plant simulation test data, abiotic transformation data such as hydrolysis and photolysis, removal process such as volatilization and finally, data obtained from field investigations and monitoring studies.

A9.4.1.2   The term degradation is defined in Chapter 4.1 as the decomposition of organic molecules to smaller molecules and eventually to carbon dioxide, water and salts. For inorganic compounds and metals, the concept of degradability as applied to organic compounds has limited or no meaning. Rather the substance may be transformed by normal environmental processes to either increase or decrease the bioavailability of the toxic species. Therefore, the present section deals only with organic substances and organo-metals. Environmental partitioning from the water column is discussed in Section A9.7.

A9.4.1.3   Data on degradation properties of a substance may be available from standardized tests or from other types of investigations, or they may be estimated from the structure of the molecules. The interpretation of such degradation data for classification purposes often requires detailed evaluation of the test data. Guidance is given in the present section and more details can be found in two paragraphs describing available methods (Appendix A9.I) and factors influencing degradation in aquatic environments (Appendix A9.II).

A9.4.2 *Interpretation of degradability data*

A9.4.2.1   *Rapid degradability*

Aquatic hazard classification of chemical substances is normally based on existing data on their environmental properties. Only seldom will test data be produced with the main purpose of facilitating a classification. Often a diverse range of test data is available that does not necessarily fits directly with the classification criteria. Consequently, guidance is needed on interpretation of existing test data in the context of the aquatic hazard classification. Based on the harmonized criteria, guidance for interpretation of degradation data is prepared below for the three types of data comprised by the expression "rapid degradation" in the aquatic environment (see A9.1.8, A9.1.9, A9.1.2.3.1 to A9.2.3.3 and the definition in Chapter 4.1, para. 4.1.2.10.3).

A9.4.2.2   *Ready biodegradability*

A9.4.2.2.1   Ready biodegradability is defined in the OECD Test Guidelines No. 301 (OECD 1992). All organic substances that degrade to a level higher than the pass level in a standard OECD ready biodegradability test or in a similar test should be considered readily biodegradable and consequently also rapidly degradable. Many literature test data, however, do not specify all of the conditions that should be evaluated to demonstrate whether or not the test fulfils the requirements of a ready biodegradability test. Expert judgement is therefore needed as regards the validity of the data before use for classification purposes. Before concluding on the ready biodegradability of a test substance, however, at least the following parameters should be considered.

A9.4.2.2.2   Concentration of test substance

Relatively high concentrations of test substance are used in the OECD ready biodegradability tests (2-100 mg/l). Many substances may, however, be toxic to the inocula at such high concentrations causing a low degradation in the tests although the substances might be rapidly degradable at lower non-toxic concentrations. A toxicity test with micro-organisms (as e.g. the OECD Test Guideline 209 "Activated Sludge, Respiration Inhibition Test", the ISO 9509 nitrification inhibition test, or the ISO 11348 luminescent bacteria inhibition test) may demonstrate the toxicity of the test substance. When it is likely that inhibition is the reason for a substance being not readily degradable, results from a test employing lower non-toxic concentrations of the test substance should be used when available. Such test results could on a case by case basis be considered in relation to the classification criteria for rapid degradation, even though surface water degradation test data with environmentally realistic microbial biomass and non toxic realistic low concentration of the test substance in general are preferred, if available.

A9.4.2.2.3   Time window

The harmonized criteria include a general requirement for all of the ready biodegradability tests on achievement of the pass level within 10 days. This is not in line with the OECD Test Guideline 301 in which the 10-days time window applies to the OECD ready biodegradability tests except to the MITI I test (OECD Test Guideline 301C). In the Closed Bottle test (OECD Test Guideline 301D), a 14-days window may be used instead when measurements have not been made after 10 days. Moreover, often only limited information is available in references of biodegradation tests. Thus, as a pragmatic approach the percentage of degradation reached after 28 days may be used directly for assessment of ready biodegradability when no information on the 10-days time window is available. This should, however, only be accepted for existing test data and data from tests where the 10-days window does not apply.

A9.4.2.3   *BOD$_5$/COD*

Information on the 5-day biochemical oxygen demand (BOD$_5$) will be used for classification purposes only when no other measured degradability data are available. Thus, priority is given to data from ready biodegradability tests and from simulation studies regarding degradability in the aquatic environment. The BOD$_5$ test is a traditional biodegradation test that is now replaced by the ready biodegradability tests. Therefore, this test should not be performed today for assessment of the ready biodegradability of substances. Older test data may, however, be used when no other degradability data are available. For substances where the chemical structure is known, the theoretical oxygen demand (ThOD) can be calculated and this value should be used instead of the chemical oxygen demand (COD).

A9.4.2.4   *Other convincing scientific evidence*

A9.4.2.4.1   Rapid degradation in the aquatic environment may be demonstrated by other data than referred to in Chapter 4.1, paragraph 4.1.2.10.3 (a) and (b). These may be data on biotic and/or abiotic degradation. Data on primary degradation can only be used where it is demonstrated that the degradation products shall not be classified as hazardous to the aquatic environment, i.e. that they do not fulfil the classification criteria.

A9.4.2.4.2   The fulfilment of paragraph 4.1.2.10.3 (c) requires that the substance is degraded in the aquatic environment to a level of >70% within a 28-day period. If first-order kinetics are assumed, which is reasonable at the low substance concentrations prevailing in most aquatic environments, the degradation rate will be relatively constant for the 28-day period. Thus, the degradation requirement will be fulfilled with an average degradation rate constant, $k > -(\ln 0.3 - \ln 1)/28 = 0.043$ day^{-1}. This corresponds to a degradation half-life, $t_{1/2} < \ln 2/0.043 = 16$ days.

A9.4.2.4.3   Moreover, as degradation processes are temperature dependent, this parameter should also be taken into account when assessing degradation in the environment. Data from studies employing environmentally realistic temperatures should be used for the evaluation. When data from studies performed at different temperatures need to be compared, the traditional Q10 approach could be used, i.e. that the degradation rate is halved when the temperature decreases by 10 °C.

A9.4.2.4.4   The evaluation of data on fulfilment of this criterion should be conducted on a case-by-case basis by expert judgement. However, guidance on the interpretation of various types of data that may be used for demonstrating a rapid degradation in the aquatic environment is given below. In general, only data from aquatic biodegradation simulation tests are considered directly applicable. However simulation test data from other environmental compartments could be considered as well, but such data require in general more scientific judgement before use.

A9.4.2.4.5   Aquatic simulation tests

Aquatic simulation tests are tests conducted in laboratory, but simulating environmental conditions and employing natural samples as inoculum. Results of aquatic simulation tests may be used directly for classification purposes, when realistic environmental conditions in surface waters are simulated, i.e.:

(a)   substance concentration that is realistic for the general aquatic environment (often in the low μg/l range);

(b)   inoculum from a relevant aquatic environment;

(c)   realistic concentration of inoculum (10^3-10^6 cells/ml);

(d)   realistic temperature (e.g. 5 °C to 25 °C); and

(e)   ultimate degradation is determined (i.e. determination of the mineralization rate or the individual degradation rates of the total biodegradation pathway).

Substances that under these conditions are degraded at least 70% within 28 days, i.e. with a half-life < 16 days, are considered rapidly degradable.

A9.4.2.4.6   Field investigations

Parallels to laboratory simulation tests are field investigations or mesocosm experiments. In such studies, fate and/or effects of chemicals in environments or environmental enclosures may be investigated. Fate data from such experiments might be used for assessing the potential for a rapid degradation. This may, however, often be difficult, as it requires that an ultimate degradation can be demonstrated. This may be documented by preparing mass balances showing that no non-degradable intermediates are formed, and which take the fractions into account that are removed from the aqueous system due to other processes such as sorption to sediment or volatilization from the aquatic environment.

A9.4.2.4.7   Monitoring data

Monitoring data may demonstrate the removal of contaminants from the aquatic environment. Such data are, however, very difficult to use for classification purposes. The following aspects should be considered before use:

(a)   Is the removal a result of degradation, or is it a result of other processes such as dilution or distribution between compartments (sorption, volatilization)?

(b)   Is formation of non-degradable intermediates excluded?

Only when it can be demonstrated that removal as a result of ultimate degradation fulfils the criteria for rapid degradability, such data be considered for use for classification purposes. In general, monitoring data should only be used as supporting evidence for demonstration of either persistence in the aquatic environment or a rapid degradation.

A9.4.2.4.8   Inherent biodegradability tests

Substances that are degraded more than 70% in tests for inherent biodegradability (OECD Test Guidelines 302) have the potential for ultimate biodegradation. However, because of the optimum conditions in these tests, the rapid biodegradability of inherently biodegradable substances in the environment cannot be assumed. The optimum conditions in inherent biodegradability tests stimulate adaptation of the micro-organisms thus increasing the biodegradation potential, compared to natural environments. Therefore, positive results in general should not be interpreted as evidence for rapid degradation in the environment[2].

A9.4.2.4.9   Sewage treatment plant simulation tests

Results from tests simulating the conditions in a sewage treatment plant (STP) (e.g. the OECD Test Guideline 303) cannot be used for assessing the degradation in the aquatic environment. The main reasons for this are that the microbial biomass in a STP is significantly different from the biomass in the environment, that there is a considerably different composition of substrates, and that the presence of rapidly mineralized organic matter in waste water facilitates degradation of the test substance by co-metabolism.

A9.4.2.4.10   Soil and sediment degradation data

It has been argued that for many non-sorptive (non-lipophilic) substances more or less the same degradation rates are found in soil and in surface water. For lipophilic substances, a lower degradation rate may generally be expected in soil than in water due to partial immobilization caused by sorption. Thus, when a substance has been shown to be degraded rapidly in a soil simulation study, it is most likely also rapidly degradable in the aquatic environment. It is therefore proposed that an experimentally determined rapid degradation in soil is sufficient documentation for a rapid degradation in surface waters when:

(a)   no pre-exposure (pre-adaptation) of the soil micro-organisms has taken place; and

(b)   an environmentally realistic concentration of substance is tested; and

(c)   the substance is ultimately degraded within 28 days with a half-life < 16 days corresponding to a degradation rate > 0.043 day^{-1}.

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[2]   *In relation to interpretation of degradation data equivalent with the harmonised OECD criteria for Chronic Category 4, the standing EU working group for environmental hazard classification of substances is discussing whether certain types of data from inherent biodegradability tests may be used in a case by case evaluation as a basis for not classifying substances otherwise fulfilling this classification criterion.*

   The inherent biodegradability tests concerned are the Zahn Wellens test (OECD TG 302 B) and the MITI II test (OECD TG 302 C). The conditions for use in this regard are:

   (a) The methods must not employ pre-exposed (pre-adapted) micro-organisms;

   (b) The time for adaptation within each test should be limited, the test endpoint should refer to the mineralization only and the pass level and time for reaching these should be, respectively:

      (i) MITI II pass level > 60 % within 14 days

      (ii) Zahn Wellens Test > 70 % within 7 days.

The same argumentation is considered valid for data on degradation in sediment under aerobic conditions.

A9.4.2.4.11   Anaerobic degradation data

Data regarding anaerobic degradation cannot be used in relation to deciding whether a substance should be regarded as rapidly degradable, because the aquatic environment is generally regarded as the aerobic compartment where the aquatic organisms, such as those employed for aquatic hazard classification, live.

A9.4.2.4.12   Hydrolysis

Data on hydrolysis (e.g. OECD Test Guideline 111) might be considered for classification purposes only when the longest half-life $t_{1/2}$ determined within the pH range 4-9 is shorter than 16 days. However, hydrolysis is not an ultimate degradation and various intermediate degradation products may be formed, some of which may be only slowly degradable. Only when it can be satisfactorily demonstrated that the hydrolysis products formed do not fulfil the criteria for classification as hazardous for the aquatic environment, data from hydrolysis studies could be considered.

When a substance is quickly hydrolysed (e.g. with $t_{1/2} <$ a few days), this process is a part of the degradation determined in biodegradation tests. Hydrolysis may be the initial transformation process in biodegradation.

A9.4.2.4.13   Photochemical degradation

Information on photochemical degradation (e.g. OECD, 1997) is difficult to use for classification purposes. The actual degree of photochemical degradation in the aquatic environment depends on local conditions (e.g. water depth, suspended solids, turbidity) and the hazard of the degradation products is usually not known. Probably only seldom will enough information be available for a thorough evaluation based on photochemical degradation.

A9.4.2.4.14   Estimation of degradation

A9.4.2.4.14.1   Certain QSARs have been developed for prediction of an approximate hydrolysis half-life, which should only be considered when no experimental data are available. However, a hydrolysis half-life can only be used in relation to classification with great care, because hydrolysis does not concern ultimate degradability (see "Hydrolysis" of this Section). Furthermore the QSARs developed until now have a rather limited applicability and are only able to predict the potential for hydrolysis on a limited number of chemical classes. The QSAR program HYDROWIN (version 1.67, Syracuse Research Corporation) is for example only able to predict the potential for hydrolysis on less than 1/5th of the existing EU substances which have a defined (precise) molecular structure (Niemelä, 2000).

A9.4.2.4.14.2   In general, no quantitative estimation method (QSAR) for estimating the degree of biodegradability of organic substances is yet sufficiently accurate to predict rapid degradation. However, results from such methods may be used to predict that a substance is not rapidly degradable. For example, when in the Biodegradation Probability Program (e.g. BIOWIN version 3.67, Syracuse Research Corporation) the probability is < 0.5 estimated by the linear or non-linear methods, the substances should be regarded as not rapidly degradable (OECD, 1994; Pedersen *et al.*, 1995 & Langenberg *et al.*, 1996). Also other (Q)SAR methods may be used as well as expert judgement, for example, when degradation data for structurally analogue compounds are available, but such judgement should be conducted with great care. In general, a QSAR prediction that a substance is not rapidly degradable is considered a better documentation for a classification than application of a default classification, when no useful degradation data are available.

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A9.4.2.4.15   Volatilization

Chemicals may be removed from some aquatic environments by volatilization. The intrinsic potential for volatilization is determined by the Henry's Law constant (H) of the substance. Volatilization from the aquatic environment is highly dependent on the environmental conditions of the specific water body in question, such as the water depth, the gas exchange coefficients (depending on wind speed and water flow) and stratification of the water body. Because volatilization only represents removal of a chemical from water phase, the Henry's Law constant cannot be used for assessment of degradation in relation to aquatic hazard classification of substances. Substances that are gases at ambient temperature may however for example be considered further in this regard (see also Pedersen *et al.*, 1995).

A9.4.2.5   *No degradation data available*

When no useful data on degradability are available - either experimentally determined or estimated data - the substance should be regarded as not rapidly degradable.

A9.4.3   *General interpretation problems*

A9.4.3.1   *Complex substances*

The harmonized criteria for classification of chemicals as hazardous for the aquatic environment focus on single substances. A certain type of intrinsically complex substance are multi-component substances. They are typically of natural origin and need occasionally to be considered. This may be the case for chemicals that are produced or extracted from mineral oil or plant material. Such complex chemicals are normally considered as single substances in a regulatory context. In most cases they are defined as a homologous series of substances within a certain range of carbon chain length and/or degree of substitution. When this is the case, no major difference in degradability is foreseen and the degree of degradability can be established from tests of the complex chemical. One exception would be when a borderline degradation is found because in this case some of the individual substances may be rapidly degradable and other may be not rapidly degradable. This requires a more detailed assessment of the degradability of the individual components in the complex substance. When not-rapidly-degradable components constitute a significant part of the complex substance (e.g. more than 20%, or for a hazardous component, an even lower content), the substance should be regarded as not rapidly degradable.

A9.4.3.2   *Availability of the substance*

A9.4.3.2.1   Degradation of organic substances in the environment takes place mostly in the aquatic compartments or in aquatic phases in soil or sediment. Hydrolysis, of course, requires the presence of water. The activity of micro-organisms depends on the presence of water. Moreover, biodegradation requires that the micro-organisms are directly in contact with the substance. Dissolution of the substance in the water phase that surrounds the micro-organisms is therefore the most direct way for contact between the bacteria and fungi and the substrate.

A9.4.3.2.2   The present standard methods for investigating degradability of chemical substances are developed for readily soluble test compounds. However, many organic substances are only slightly soluble in water. As the standard tests require 2-100 mg/l of the test substance, sufficient availability may not be reached for substances with a low water solubility. Tests with continuous mixing and/or an increased exposure time, or tests with a special design where concentrations of the test substance lower than the water solubility have been employed, may be available on slightly soluble compounds.

A9.4.3.3   *Test duration less than 28 days*

A9.4.3.3.1   Sometimes degradation is reported for tests terminated before the 28 day period specified in the standards (e.g. the MITI, 1992). These data are of course directly applicable when a degradation greater than or equal to the pass level is obtained. When a lower degradation level is reached, the results need to be interpreted with caution. One possibility is that the duration of the test was too short and that the chemical

structure would probably have been degraded in a 28-day biodegradability test. If substantial degradation occurs within a short time period, the situation may be compared with the criterion $BOD_5/COD \geq 0.5$ or with the requirements on degradation within the 10-days time window. In these cases, a substance may be considered readily degradable (and hence rapidly degradable), if:

       (a)   the ultimate biodegradability > 50% within 5 days; or

       (b)   the ultimate degradation rate constant in this period is > 0.1 day^{-1} corresponding to a half-life of 7 days.

A9.4.3.3.2   These criteria are proposed in order to ensure that rapid mineralization did occur, although the test was ended before 28 days and before the pass level was attained. Interpretation of test data that do not comply with the prescribed pass levels must be made with great caution. It is mandatory to consider whether a biodegradability below the pass level was due to a partial degradation of the substance and not a complete mineralization. If partial degradation is the probable explanation for the observed biodegradability, the substance should be considered not readily biodegradable.

A9.4.3.4   *Primary biodegradation*

       In some tests, only the disappearance of the parent compound (i.e. primary degradation) is determined for example by following the degradation by specific or group specific chemical analyses of the test substance. Data on primary biodegradability may be used for demonstrating rapid degradability only when it can be satisfactorily demonstrated that the degradation products formed do not fulfil the criteria for classification as hazardous to the aquatic environment.

A9.4.3.5   *Conflicting results from screening tests*

A9.4.3.5.1   The situation where more degradation data are available for the same substance introduces the possibility of conflicting results. In general, conflicting results for a substance which has been tested several times with an appropriate biodegradability test could be interpreted by a "weight of evidence approach". This implies that if both positive (i.e. higher degradation than the pass level) and negative results have been obtained for a substance in ready biodegradability tests, then the data of the highest quality and the best documentation should be used for determining the ready biodegradability of the substance. However, positive results in ready biodegradability tests could be considered valid, irrespective of negative results, when the scientific quality is good and the test conditions are well documented, i.e. guideline criteria are fulfilled, including the use of non-pre-exposed (non-adapted) inoculum. None of the various screening tests are suitable for the testing of all types of substances, and results obtained by the use of a test procedure which is not suitable for the specific substance should be evaluated carefully before a decision on the use is taken.

A9.4.3.5.2   Thus, there are a number of factors that may explain conflicting biodegradability data from screening tests:

       (a)   inoculum;

       (b)   toxicity of test substance;

       (c)   test conditions;

       (d)   solubility of the test substance; and

       (e)   volatilization of the test substance.

A9.4.3.5.3   The suitability of the inoculum for degrading the test substance depends on the presence and amount of competent degraders. When the inoculum is obtained from an environment that has previously been exposed to the test substance, the inoculum may be adapted as evidenced by a degradation capacity, which is greater than that of an inoculum from a non-exposed environment. As far as possible the inoculum

must be sampled from an unexposed environment, but for substances that are used ubiquitously in high volumes and released widespread or more or less continuously, this may be difficult or impossible. When conflicting results are obtained, the origin of the inoculum should be checked in order to clarify whether or not differences in the adaptation of the microbial community may be the reason.

A9.4.3.5.4   As mentioned above, many substances may be toxic or inhibitory to the inoculum at the relatively high concentrations tested in ready biodegradability tests. Especially in the Modified MITI (I) test (OECD Test Guideline 301C) and the Manometric Respirometry test (OECD Test Guideline 301F) high concentrations (100 mg/l) are prescribed. The lowest test substance concentrations are prescribed in the Closed Bottle test (OECD Test Guideline 301D) where 2-10 mg/l is used. The possibility of toxic effects may be evaluated by including a toxicity control in the ready biodegradability test or by comparing the test concentration with toxicity test data on micro-organisms, e.g. the respiration inhibition tests (OECD Test Guideline 209), the nitrification inhibition test (ISO 9509) or, if other microbial toxicity tests are not available, the bioluminescence inhibition test (ISO 11348). When conflicting results are found, this may becaused by toxicity of the test substance. If the substance is not inhibitory at environmentally realistic concentrations, the greatest degradation measured in screening tests may be used as a basis for classification. If simulation test data are available in such cases, consideration of these data may be especially important, because a low non inhibitory concentration of the substance may have been employed, thus giving a more reliable indication of the biodegradation half-life of the substance under environmentally realistic conditions.

A9.4.3.5.5   When the solubility of the test substance is lower than the concentrations employed in a test, this parameter may be the limiting factor for the actual degradation measured. In these cases, results from tests employing the lowest concentrations of test substance should prevail, i.e. often the Closed Bottle test (OECD Test Guideline 301D). In general, the DOC Die-Away test (OECD Test Guideline 301A) and the Modified OECD Screening test (OECD Test Guideline 301E) are not suitable for testing the biodegradability of poorly soluble substances (e.g. OECD Test Guideline 301).

A9.4.3.5.6   Volatile substances should only be tested in closed systems as the Closed Bottle test (OECD Test Guideline 301D), the MITI I test (OECD Test Guideline 301C) and the Manometric Respirometry test (OECD Test Guideline 301F). Results from other tests should be evaluated carefully and only considered if it can be demonstrated, e.g. by mass balance estimates, that the removal of the test substance is not a result of volatilization.

A9.4.3.6   *Variation in simulation test data*

A number of simulation test data may be available for certain high priority chemicals. Often such data provide a range of half lives in environmental media such as soil, sediment and/or surface water. The observed differences in half-lives from simulation tests performed on the same substance may reflect differences in test conditions, all of which may be environmentally relevant. A suitable half life in the higher end of the observed range of half lives from such investigations should be selected for classification by employing a weight of evidence approach and taking the realism and relevance of the employed tests into account in relation to environmental conditions. In general, simulation test data of surface water are preferred relative to aquatic sediment or soil simulation test data in relation to the evaluation of rapid degradability in the aquatic environment.

A9.4.4   *Decision scheme*

The following decision scheme may be used as a general guidance to facilitate decisions in relation to rapid degradability in the aquatic environment and classification of chemicals hazardous to the aquatic environment.

A substance is considered to be not rapidly degradable unless at least one of the following is fulfilled:

(a)   the substance is demonstrated to be readily biodegradable in a 28-day test for ready biodegradability. The pass level of the test (70% DOC removal or 60% theoretical oxygen demand) must be achieved within 10 days from the onset of biodegradation, if it is possible to evaluate this according to the available test data. If this is not possible, then the pass level should be evaluated within a 14 days time window if possible, or after the end of the test; or

(b)   the substance is demonstrated to be ultimately degraded in a surface water simulation test[3] with a half-life of < 16 days (corresponding to a degradation of > 70% within 28 days); or

(c)   the substance is demonstrated to be primarily degraded (biotically or abiotically) in the aquatic environment with a half-life < 16 days (corresponding to a degradation of > 70% within 28 days) and it can be demonstrated that the degradation products do not fulfil the criteria for classification as hazardous to the aquatic environment.

When these data are not available rapid degradation may be demonstrated if either of the following criteria are justified:

(d)   the substance is demonstrated to be ultimately degraded in an aquatic sediment or soil simulation test[3] with a half-life of < 16 days (corresponding to a degradation of > 70% within 28 days); or

(e)   in those cases where only BOD_5 and COD data are available, the ratio of BOD_5/COD is ≥ 0.5. The same criterion applies to ready biodegradability tests of a shorter duration than 28 days, if the half-life furthermore is < 7 days.

If none of the above types of data are available then the substance is considered as not rapidly degradable. This decision may be supported by fulfilment of at least one of the following criteria:

(i)   the substance is not inherently degradable in an inherent biodegradability test; or

(ii)   the substances is predicted to be slowly biodegradable by scientifically valid QSARs, e.g. for the Biodegradation Probability Program, the score for rapid degradation (linear or non-linear model) < 0.5; or

(iii)   the substance is considered to be not rapidly degradable based on indirect evidence, as e.g. knowledge from structurally similar substances; or

(iv)   no other data regarding degradability are available.

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[3]   *Simulations tests should reflect realistic environmental conditions such as low concentration of the chemical, realistic temperature and employment of ambient microbial biomass not pre-exposed to the chemical.*

A9.**5** **Bioaccumulation**

A9.5.1 *Introduction*

A9.5.1.1   Bioaccumulation is one of the important intrinsic properties of chemical substances that determine the potential environmental hazard. Bioaccumulation of a substance into an organism is not a hazard in itself, but bioconcentration and bioaccumulation will result in a body burden, which may or may not lead to toxic effects. In the harmonized integrated hazard classification system for human health and environmental effects of chemical substances (OECD, 1998), the wording "potential for bioaccumulation" is given. A distinction should, however, be drawn between bioconcentration and bioaccumulation. Here bioconcentration is defined as the net result of uptake, transformation, and elimination of a substance in an organism due to waterborne exposure, whereas bioaccumulation includes all routes of exposure (i.e. via air, water, sediment/soil, and food). Finally, biomagnification is defined as accumulation and transfer of substances via the food chain, resulting in an increase of internal concentrations in organisms on higher levels of the trophic chain (European Commission, 1996). For most organic chemicals uptake from water (bioconcentration) is believed to be the predominant route of uptake. Only for very hydrophobic substances does uptake from food becomes important. Also, the harmonized classification criteria use the bioconcentration factor (or the octanol/water partition coefficient) as the measure of the potential for bioaccumulation. For these reasons, the present guidance document only considers bioconcentration and does not discuss uptake via food or other routes.

A9.5.1.2   Classification of a chemical substance is primarily based on its intrinsic properties. However, the degree of bioconcentration also depends on factors such as the degree of bioavailability, the physiology of test organism, maintenance of constant exposure concentration, exposure duration, metabolism inside the body of the target organism and excretion from the body. The interpretation of the bioconcentration potential in a chemical classification context therefore requires an evaluation of the intrinsic properties of the substance, as well as of the experimental conditions under which bioconcentration factor (BCF) has been determined. Based on the guide, a decision scheme for application of bioconcentration data or log K_{ow} data for classification purposes has been developed. The emphasis of the present section is organic substances and organo-metals. Bioaccumulation of metals is also discussed in Section A9.7.

A9.5.1.3   Data on bioconcentration properties of a substance may be available from standardized tests or may be estimated from the structure of the molecule. The interpretation of such bioconcentration data for classification purposes often requires detailed evaluation of test data. In order to facilitate this evaluation two additional appendixes are enclosed. These appendixes describe available methods (Appendix III of Annex 9) and factors influencing the bioconcentration potential (Appendix IV of Annex 9). Finally, a list of standardized experimental methods for determination of bioconcentration and K_{ow} are attached (Appendix V of Annex 9) together with a list of references (Appendix VI of Annex 9).

A9.5.2 *Interpretation of bioconcentration data*

A9.5.2.1   Environmental hazard classification of a chemical substance is normally based on existing data on its environmental properties. Test data will only seldom be produced with the main purpose of facilitating a classification. Often a diverse range of test data is available which does not necessarily match the classification criteria. Consequently, guidance is needed on interpretation of existing test data in the context of hazard classification.

A9.5.2.2   Bioconcentration of an organic substance can be experimentally determined in bioconcentration experiments, during which BCF is measured as the concentration in the organism relative to the concentration in water under steady-state conditions and/or estimated from the uptake rate constant (k_1) and the elimination rate constant (k_2) (OECD 305, 1996). In general, the potential of an organic substance to bioconcentrate is primarily related to the lipophilicity of the substance. A measure of lipophilicity is the n-octanol-water partition coefficient (K_{ow}) which, for lipophilic non-ionic organic substances, undergoing minimal metabolism or biotransformation within the organism, is correlated with the bioconcentration factor. Therefore, K_{ow} is often used for estimating the bioconcentration of organic substances, based on the empirical relationship between log BCF and log K_{ow}. For most organic substances, estimation methods are

available for calculating the K_{ow}. Data on the bioconcentration properties of a substance may thus be (i) experimentally determined, (ii) estimated from experimentally determined K_{ow}, or (iii) estimated from K_{ow} values derived by use of Quantitative Structure Activity Relationships (QSARs). Guidance for interpretation of such data is given below together with guidance on assessment of chemical classes, which need special attention.

A9.5.2.3   *Bioconcentration factor (BCF)*

A9.5.2.3.1   The bioconcentration factor is defined as the ratio on a weight basis between the concentration of the chemical in biota and the concentration in the surrounding medium, here water, at steady state. BCF can thus be experimentally derived under steady-state conditions, on the basis of measured concentrations. However, BCF can also be calculated as the ratio between the first-order uptake and elimination rate constants; a method which does not require equilibrium conditions.

A9.5.2.3.2   Different test guidelines for the experimental determination of bioconcentration in fish have been documented and adopted, the most generally applied being the OECD test guideline (OECD 305, 1996).

A9.5.2.3.3   Experimentally derived BCF values of high quality are ultimately preferred for classification purposes as such data override surrogate data, e.g. K_{ow}.

A9.5.2.3.4   High quality data are defined as data where the validity criteria for the test method applied are fulfilled and described, e.g. maintenance of constant exposure concentration; oxygen and temperature variations, and documentation that steady-state conditions have been reached, etc. The experiment will be regarded as a high-quality study, if a proper description is provided (e.g. by Good Laboratory Practice (GLP)) allowing verification that validity criteria are fulfilled. In addition, an appropriate analytical method must be used to quantify the chemical and its toxic metabolites in the water and fish tissue (see section 1, Appendix III for further details).

A9.5.2.3.5   BCF values of low or uncertain quality may give a false and too low BCF value; e.g. application of measured concentrations of the test substance in fish and water, but measured after a too short exposure period in which steady-state conditions have not been reached (cf. OECD 306, 1996, regarding estimation of time to equilibrium). Therefore, such data should be carefully evaluated before use and consideration should be given to using K_{ow} instead.

A9.5.2.3.6   If there is no BCF value for fish species, high-quality data on the BCF value for other species may be used (e.g. BCF determined on blue mussel, oyster, scallop (ASTM E 1022-94)). Reported BCFs for microalgae should be used with caution.

A9.5.2.3.7   For highly lipophilic substances, e.g. with log K_{ow} above 6, experimentally derived BCF values tend to decrease with increasing log K_{ow}. Conceptual explanations of this non-linearity mainly refer to either reduced membrane permeation kinetics or reduced biotic lipid solubility for large molecules. A low bioavailability and uptake of these substances in the organism will thus occur. Other factors comprise experimental artefacts, such as equilibrium not being reached, reduced bioavailability due to sorption to organic matter in the aqueous phase, and analytical errors. Special care should thus be taken when evaluating experimental data on BCF for highly lipophilic substances as these data will have a much higher level of uncertainty than BCF values determined for less lipophilic substances.

A9.5.2.3.8   BCF in different test species

A9.5.2.3.8.1   BCF values used for classification are based on whole body measurements. As stated previously, the optimal data for classification are BCF values derived using the OECD 305 test method or internationally equivalent methods, which uses small fish. Due to the higher gill surface to weight ratio for smaller organisms than larger organisms, steady-state conditions will be reached sooner in smaller organisms than in larger ones. The size of the organisms (fish) used in bioconcentration studies is thus of considerable importance in relation to the time used in the uptake phase, when the reported BCF value is based solely on

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measured concentrations in fish and water at steady-state. Thus, if large fish, e.g. adult salmon, have been used in bioconcentration studies, it should be evaluated whether the uptake period was sufficiently long for steady state to be reached or to allow for a kinetic uptake rate constant to be determined precisely.

A9.5.2.3.8.2   Furthermore, when using existing data for classification, it is possible that the BCF values could be derived from several different fish or other aquatic species (e.g. clams) and for different organs in the fish. Thus, to compare these data to each other and to the criteria, some common basis or normalization will be required. It has been noted that there is a close relationship between the lipid content of a fish or an aquatic organism and the observed BCF value. Therefore, when comparing BCF values across different fish species or when converting BCF values for specific organs to whole body BCFs, the common approach is to express the BCF values on a common lipid content. If e.g. whole body BCF values or BCF values for specific organs are found in the literature, the first step is to calculate the BCF on a % lipid basis using the relative content of fat in the fish (cf. literature/test guideline for typical fat content of the test species) or the organ. In the second step the BCF for the whole body for a typical aquatic organism (i.e. small fish) is calculated assuming a common default lipid content. A default value of 5% is most commonly used (Pedersen *et al.*, 1995) as this represents the average lipid content of the small fish used in OECD 305 (1996).

A9.5.2.3.8.3   Generally, the highest valid BCF value expressed on this common lipid basis is used to determine the wet weight based BCF-value in relation to the cut off value for BCF of 500 of the harmonized classification criteria (see Chapter 4.1, Figure 4.1.1).

A9.5.2.3.9   Use of radiolabelled substances

A9.5.2.3.9.1   The use of radiolabelled test substances can facilitate the analysis of water and fish samples. However, unless combined with a specific analytical method, the total radioactivity measurements potentially reflect the presence of the parent substance as well as possible metabolite(s) and possible metabolized carbon, which have been incorporated in the fish tissue in organic molecules. BCF values determined by use of radiolabelled test substances are therefore normally overestimated.

A9.5.2.3.9.2   When using radiolabelled substances, the labelling is most often placed in the stable part of the molecule, for which reason the measured BCF value includes the BCF of the metabolites. For some substances it is the metabolite which is the most toxic and which has the highest bioconcentration potential. Measurements of the parent substance as well as the metabolites may thus be important for the interpretation of the aquatic hazard (including the bioconcentration potential) of such substances.

A9.5.2.3.9.3   In experiments where radiolabelled substances have been used, high radiolabel concentrations are often found in the gall bladder of fish. This is interpreted to be caused by biotransformation in the liver and subsequently by excretion of metabolites in the gall bladder (Comotto *et al.*, 1979; Wakabayashi *et al.*, 1987; Goodrich *et al.*, 1991; Toshima *et al.*, 1992). When fish do not eat, the content of the gall bladder is not emptied into the gut, and high concentrations of metabolites may build up in the gall bladder. The feeding regime may thus have a pronounced effect on the measured BCF. In the literature many studies are found where radiolabelled compounds are used, and where the fish are not fed. As a result high concentrations of radioactive material are found in the gall bladder. In these studies the bioconcentration may in most cases have been overestimated. Thus when evaluating experiments, in which radiolabelled compounds are used, it is essential to evaluate the feeding regime as well.

A9.5.2.3.9.4   If the BCF in terms of radiolabelled residues is documented to be ≥ 1000, identification and quantification of degradation products, representing $\geq 10\%$ of total residues in fish tissues at steady-state, are for e.g. pesticides strongly recommended in the OECD guideline No. 305 (1996). If no identification and quantification of metabolites are available, the assessment of bioconcentration should be based on the measured radiolabelled BCF value. If, for highly bioaccumulative substances (BCF ≥ 500), only BCFs based on the parent compound and on radiolabelled measurements are available, the latter should thus be used in relation to classification.

A9.5.2.4　　　　*Octanol-water-partitioning coefficient (K_{ow})*

A9.5.2.4.1　　　　For organic substances experimentally derived high-quality K_{ow} values, or values which are evaluated in reviews and assigned as the "recommended values", are preferred over other determinations of Kow. When no experimental data of high quality are available, validated Quantitative Structure Activity Relationships (QSARs) for log K_{ow} may be used in the classification process. Such validated QSARs may be used without modification to the agreed criteria if they are restricted to chemicals for which their applicability is well characterized. For substances like strong acids and bases, substances which react with the eluent, or surface-active substances, a QSAR estimated value of K_{ow} or an estimate based on individual *n*-octanol and water solubilities should be provided instead of an analytical determination of K_{ow} (EEC A.8., 1992; OECD 117, 1989). Measurements should be taken on ionizable substances in their non-ionized form (free acid or free base) only by using an appropriate buffer with pH below pK for free acid or above the pK for free base.

A9.5.2.4.2　　　　Experimental determination of K_{ow}

　　　　　　For experimental determination of K_{ow} values, several different methods, Shake-flask, and HPLC, are described in standard guidelines, e.g. OECD Test Guideline 107 (1995); OECD Test Guideline 117 (1989); EEC A.8. (1992); EPA-OTS (1982); EPA-FIFRA (1982); ASTM (1993); the pH-metric method (OECD Test Guideline in preparation). The shake-flask method is recommended when the log K_{ow} value falls within the range from –2 to 4. The shake-flask method applies only to essential pure substances soluble in water and *n*-octanol. For highly lipophilic substances, which slowly dissolve in water, data obtained by employing a slow-stirring method are generally more reliable. Furthermore, the experimental difficulties, associated with the formation of microdroplets during the shake-flask experiment, can to some degree be overcome by a slow-stirring method where water, octanol, and test compound are equilibrated in a gently stirred reactor. With the slow-stirring method (OECD Test Guideline in preparation) a precise and accurate determination of K_{ow} of compounds with log K_{ow} of up to 8.2 is allowed (OECD draft Guideline, 1998). As for the shake-flask method, the slow-stirring method applies only to essentially pure substances soluble in water and *n*-octanol. The HPLC method, which is performed on analytical columns, is recommended when the log K_{ow} value falls within the range 0 to 6. The HPLC method is less sensitive to the presence of impurities in the test compound compared to the shake-flask method. Another technique for measuring log K_{ow} is the generator column method (USEPA 1985).

　　　　　　As an experimental determination of the K_{ow} is not always possible, e.g. for very water-soluble substances, very lipophilic substances, and surfactants, a QSAR-derived K_{ow} may be used.

A9.5.2.4.3　　　　Use of QSARs for determination of log K_{ow}

　　　　　　When an estimated K_{ow} value is found, the estimation method has to be taken into account. Numerous QSARs have been and continue to be developed for the estimation of K_{ow}. Four commercially available PC programmes (CLOGP, LOGKOW (KOWWIN), AUTOLOGP, SPARC) are frequently used for risk assessment if no experimentally derived data are available. CLOGP, LOGKOW and AUTOLOGP are based upon the addition of group contributions, while SPARC is based upon a more fundamental chemical structure algorithm. Only SPARC can be employed in a general way for inorganic or organometallic compounds. Special methods are needed for estimating log K_{ow} for surface-active compounds, chelating compounds and mixtures. CLOGP is recommended in the US EPA/EC joint project on validation of QSAR estimation methods (US EPA/EC 1993). Pedersen *et al*. (1995) recommended the CLOGP and the LOGKOW programmes for classification purposes because of their reliability, commercial availability, and convenience of use. The following estimation methods are recommended for classification purposes (Table A9.5.1).

Table A9.5.1: Recommended QSARs for estimation of K_{ow}

Model	log K_{ow} range	Substance utility
CLOGP	$0 < \log K_{ow} < 9$ [a]	The program calculates log K_{ow} for organic compounds containing C, H, N, O, Hal, P, and/or S.
LOGKOW (KOWWIN)	$-4 < \log K_{ow} < 8$ [b]	The program calculates log K_{ow} for organic compounds containing C, H, N, O, Hal, Si, P, Se, Li, Na, K, and/or Hg. Some surfactants (e.g. alcohol ethoxylates, dyestuffs, and dissociated substances may be predicted by the program as well.
AUTOLOGP	$\log K_{ow} > 5$	The programme calculates log K_{ow} for organic compounds containing C, H, N, O, Hal, P and S. Improvements are in progress in order to extend the applicability of AUTOLOGP.
SPARC	Provides improved results over KOWWIN and CLOGP for compounds with $\log K_{ow} > 5$	SPARC is a mechanistic model based on chemical thermodynamic principles rather than a deterministic model rooted in knowledge obtained from observational data. Therefore, SPARC differs from models that use QSARs (i.e. KOWWIN, CLOGP, AUTOLOGP) in that no measured log K_{ow} data are needed for a training set of chemicals. Only SPARC can be employed in a general way for inorganic or organometallic compounds.

[a]   *A validation study performed by Niemelä, who compared experimental determined log K_{ow} values with estimated values, showed that the program precisely predicts the log K_{ow} for a great number of organic chemicals in the log K_{ow} range from below 0 to above 9 (n = 501, r^2 = 0.967) (TemaNord 1995: 581).*

[b]   *Based on a scatter plot of estimated vs. experimental log K_{ow} (Syracuse Research Corporation, 1999), where 13058 compound have been tested, the LOGKOW is evaluated being valid for compounds with a log K_{ow} in the interval -4 - 8.*

A9.5.3   ***Chemical classes that need special attention with respect to BCF and K_{ow} values***

A9.5.3.1   There are certain physico-chemical properties, which can make the determination of BCF or its measurement difficult. These may be substances, which do not bioconcentrate in a manner consistent with their other physico-chemical properties, e.g. steric hindrance or substances which make the use of descriptors inappropriate, e.g. surface activity, which makes both the measurement and use of log K_{ow} inappropriate.

A9.5.3.2   *Difficult substances*

A9.5.3.2.1   Some chemical substances are difficult to test in aquatic systems and guidance has been developed to assist in testing these materials (DoE, 1996; ECETOC 1996; and US EPA 1996). OECD is in the process of finalizing a guidance document for the aquatic testing of difficult substances (OECD, 2000). This latter document is a good source of information, also for bioconcentration studies, on the types of substances that are difficult to test and the steps needed to ensure valid conclusions from tests with these substances. Difficult to test substances may be poorly soluble, volatile, or subject to rapid degradation due to such processes as phototransformation, hydrolysis, oxidation, or biotic degradation.

A9.5.3.2.2   To bioconcentrate organic compounds, a substance needs to be soluble in lipids, present in the water, and available for transfer across the fish gills. Properties which alter this availability will thus change the actual bioconcentration of a substance, when compared with the prediction. For example, readily biodegradable substances may only be present in the aquatic compartment for short periods of time. Similarly, volatility, and hydrolysis will reduce the concentration and the time during which a substance is available for bioconcentration. A further important parameter, which may reduce the actual exposure concentration of a substance, is adsorption, either to particulate matter or to surfaces in general. There are a number of substances, which have shown to be rapidly transformed in the organism, thus leading to a lower BCF value than expected. Substances that form micelles or aggregates may bioconcentrate to a lower extent

than would be predicted from simple physico-chemical properties. This is also the case for hydrophobic substances that are contained in micelles formed as a consequence of the use of dispersants. Therefore, the use of dispersants in bioaccumulation tests is discouraged.

A9.5.3.2.3   In general, for difficult to test substances, measured BCF and K_{ow} values – based on the parent substance – are a prerequisite for the determination of the bioconcentration potential. Furthermore, proper documentation of the test concentration is a prerequisite for the validation of the given BCF value.

A9.5.3.3   *Poorly soluble and complex substances*

Special attention should be paid to poorly soluble substances. Frequently the solubility of these substances is recorded as less than the detection limit, which creates problems in interpreting the bioconcentration potential. For such substances the bioconcentration potential should be based on experimental determination of log K_{ow} or QSAR estimations of log K_{ow}.

When a multi-component substance is not fully soluble in water, it is important to attempt to identify the components of the mixture as far as practically possible and to examine the possibility of determining its bioaccumulation potential using available information on its components. When bioaccumulating components constitute a significant part of the complex substance (e.g. more than 20% or for hazardous components an even lower content), the complex substance should be regarded as being bioaccumulating.

A9.5.3.4   *High molecular weight substances*

Above certain molecular dimensions, the potential of a substance to bioconcentrate decreases.. This is possibly due to steric hindrance of the passage of the substance through gill membranes. It has been proposed that a cut-off limit of 700 for the molecular weight could be applied (e.g. European Commission, 1996). However, this cut-off has been subject to criticism and an alternative cut-off of 1000 has been proposed in relation to exclusion of consideration of substances with possible indirect aquatic effects (CSTEE, 1999). In general, bioconcentration of possible metabolites or environmental degradation products of large molecules should be considered. Data on bioconcentration of molecules with a high molecular weight should therefore be carefully evaluated and only be used if such data are considered to be fully valid in respect to both the parent compound and its possible metabolites and environmental degradation products.

A9.5.3.5   *Surface-active agents*

A9.5.3.5.1   Surfactants consist of a lipophilic (most often an alkyl chain) and a hydrophilic part (the polar headgroup). According to the charge of the headgroup, surfactants are subdivided into classes of anionic, cationic, non-ionic, or amphoteric surfactants. Due to the variety of different headgroups, surfactants are a structurally diverse class of compounds, which is defined by surface activity rather than by chemical structure. The bioaccumulation potential of surfactants should thus be considered in relation to the different subclasses (anionic, cationic, non-ionic, or amphoteric) instead of to the group as a whole. Surface-active substances may form emulsions, in which the bioavailability is difficult to ascertain. Micelle formation can result in a change of the bioavailable fraction even when the solutions are apparently formed, thus giving problems in interpretation of the bioaccumulation potential**.**

A9.5.3.5.2   Experimentally derived bioconcentration factors

Measured BCF values on surfactants show that BCF may increase with increasing alkyl chain length and be dependant of the site of attachment of the head group, and other structural features.

A9.5.3.5.3   Octanol-water-partition coefficient (K_{ow})

The octanol-water partition coefficient for surfactants can not be determined using the shake-flask or slow stirring method because of the formation of emulsions. In addition, the surfactant molecules will exist in the water phase almost exclusively as ions, whereas they will have to pair with a counter-ion in

order to be dissolved in octanol. Therefore, experimental determination of K_{ow} does not characterize the partition of ionic surfactants (Tolls, 1998). On the other hand, it has been shown that the bioconcentration of anionic and non-ionic surfactants increases with increasing lipophilicity (Tolls, 1998). Tolls (1998) showed that for some surfactants, an estimated log K_{ow} value using LOGKOW could represent the bioaccumulation potential; however, for other surfactants some 'correction' to the estimated log K_{ow} value using the method of Roberts (1989) was required. These results illustrate that the quality of the relationship between log K_{ow} estimates and bioconcentration depends on the class and specific type of surfactants involved. Therefore, the classification of the bioconcentration potential based on log K_{ow} values should be used with caution.

A9.5.4 *Conflicting data and lack of data*

A9.5.4.1 *Conflicting BCF data*

In situations where multiple BCF data are available for the same substance, the possibility of conflicting results might arise. In general, conflicting results for a substance, which has been tested several times with an appropriate bioconcentration test, should be interpreted by a "weight of evidence approach". This implies that if experimental determined BCF data, both ≥ and < 500, have been obtained for a substance the data of the highest quality and with the best documentation should be used for determining the bioconcentration potential of the substance. If differences still remain, if e.g. high-quality BCF values for different fish species are available, generally the highest valid value should be used as the basis for classification.

When larger data sets (4 or more values) are available for the same species and life stage, the geometric mean of the BCF values may be used as the representative BCF value for that species.

A9.5.4.2 *Conflicting log K_{ow} data*

The situations, where multiple log K_{ow} data are available for the same substance, the possibility of conflicting results might arise. If log K_{ow} data both ≥ and < 4 have been obtained for a substance, then the data of the highest quality and the best documentation should be used for determining the bioconcentration potential of the substance. If differences still exist, generally the highest valid value should take precedence. In such situation, QSAR estimated log K_{ow} could be used as a guidance.

A9.5.4.3 *Expert judgement*

If no experimental BCF or log K_{ow} data or no predicted log K_{ow} data are available, the potential for bioconcentration in the aquatic environment may be assessed by expert judgement. This may be based on a comparison of the structure of the molecule with the structure of other substances for which experimental bioconcentration or log K_{ow} data or predicted K_{ow} are available.

A9.5.5 *Decision scheme*

A9.5.5.1 Based on the above discussions and conclusions, a decision scheme has been elaborated which may facilitate decisions as to whether or not a substance has the potential for bioconcentration in aquatic species.

A9.5.5.2 Experimentally derived BCF values of high quality are ultimately preferred for classification purposes. BCF values of low or uncertain quality should not be used for classification purposes if data on log K_{ow} are available because they may give a false and too low BCF value, e.g. due to a too short exposure period in which steady-state conditions have not been reached. If no BCF is available for fish species, high quality data on the BCF for other species (e.g. mussels) may be used.

A9.5.5.3 For organic substances, experimentally derived high quality K_{ow} values, or values which are evaluated in reviews and assigned as the "recommended values", are preferred. If no experimentally data of high quality are available validated Quantitative Structure Activity Relationships (QSARs) for log K_{ow} may be used in the classification process. Such validated QSARs may be used without modification in relation to

the classification criteria, if restricted to chemicals for which their applicability is well characterized. For substances like strong acids and bases, metal complexes, and surface-active substances a QSAR estimated value of K_{ow} or an estimate based on individual *n*-octanol and water solubilities should be provided instead of an analytical determination of K_{ow}.

A9.5.5.4   If data are available but not validated, expert judgement should be used.

A9.5.5.5   Whether or not a substance has a potential for bioconcentration in aquatic organisms could thus be decided in accordance with the following scheme:

> (a)   Valid/high quality experimentally determined BCF value = YES:
>
> > (i)   BCF $\geq$ 500: *The substance has a potential for bioconcentration*
> > (ii)   BCF < 500: *The substance does not have a potential for bioconcentration.*
>
> (b)   Valid/high quality experimentally determined BCF value = NO:
>
> Valid/high quality experimentally determined log K_{ow} value =YES:
> > (ii)   log K_{ow} $\geq$ 4: *The substance has a potential for bioconcentration*
> > (iii) log K_{ow} < 4: *The substance does not have a potential for bioconcentration.*
>
> (c)   Valid/high quality experimentally determined BCF value = NO:
>
> Valid/high quality experimentally determined log K_{ow} value =NO:
> Use of validated QSAR for estimating a log K_{ow} value = YES:
> > (iii) log K_{ow} $\geq$ 4: *The substance has a potential for bioconcentration*
> > (iv)   log K_{ow} < 4: *The substance does not have a potential for bioconcentration.*

A9.6   Use of QSAR

A9.6.1   *History*

A9.6.1.1   Quantitative Structure-Activity Relationships (QSAR) in aquatic toxicology can be traced to the work of Overton in Zürich (Lipnick, 1986) and Meyer in Marburg (Lipnick, 1989a). They demonstrated that the potency of substances producing narcosis in tadpoles and small fish is in direct proportion to their partition coefficients measured between olive oil and water. Overton postulated in his 1901 monograph "Studien über die Narkose," that this correlation reflects toxicity taking place at a standard molar concentration or molar volume within some molecular site within the organism (Lipnick, 1991a). In addition, he concluded that this corresponds to the same concentration or volume for a various organisms, regardless of whether uptake is from water or via gaseous inhalation. This correlation became known in anaesthesia as the Meyer-Overton theory.

A9.6.1.2   Corwin Hansch and co-workers at Pomona College proposed the use of n-octanol/water as a standard partitioning system, and found that these partition coefficients were an additive, constitutive property that can be directly estimated from chemical structure. In addition, they found that regression analysis could be used to derive QSAR models, providing a statistical analysis of the findings. Using this approach, in 1972 these workers reported 137 QSAR models in the form log (1/C) = A log K_{ow} + B, where K_{ow} is the n-octanol/water partition coefficient, and C is the molar concentration of a chemical yielding a standard biological response for the effect of simple non-electrolyte non-reactive organic compounds on whole animals, organs, cells, or even pure enzymes. Five of these equations, which relate to the toxicity of five simple monohydric alcohols to five species of fish, have almost identical slopes and intercepts that are in fact virtually the same as those found by Könemann in 1981, who appears to have been unaware of Hansch's earlier work. Könemann and others have demonstrated that such simple non-reactive non-electrolytes all

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act by a narcosis mechanism in an acute fish toxicity test, giving rise to minimum or baseline toxicity (Lipnick, 1989b).

A9.6.2 *Experimental artifacts causing underestimation of hazard*

A9.6.2.1   Other non-electrolytes can be more toxic than predicted by such a QSAR, but not less toxic, except as a result of a testing artefact. Such testing artefacts include data obtained for compounds such as hydrocarbons which tend to volatilize during the experiment, as well as very hydrophobic compounds for which the acute testing duration may be inadequate to achieve steady state equilibrium partitioning between the concentration in the aquatic phase (aquarium test solution), and the internal hydrophobic site of narcosis action. A QSAR plot of log K_{ow} vs log C for such simple non-reactive non-electrolytes exhibits a linear relationship so long as such equilibrium is established within the test duration. Beyond this point, a bilinear relationship is observed, with the most toxic chemical being the one with the highest log K_{ow} value for which such equilibrium is established (Lipnick, 1995).

A9.6.2.2   Another testing problem is posed by water solubility cut-off. If the toxic concentration required to produce the effect is above the compound's water solubility, no effect will be observed even at water saturation. Compounds for which the predicted toxic concentration is close to water solubility will also show no effect if the test duration is insufficient to achieve equilibrium partitioning. A similar cut-off is observed for surfactants if toxicity is predicted at a concentration beyond the critical micelle concentration. Although such compounds may show no toxicity under these conditions when tested alone, their toxic contributions to mixtures are still present. For compounds with the same log K_{ow} value, differences in water solubility reflect differences in enthalpy of fusion related to melting point. Melting point is a reflection of the degree of stability of the crystal lattice and is controlled by intermolecular hydrogen bonding, lack of conformational flexibility, and symmetry. The more highly symmetric a compound, the higher the melting point (Lipnick, 1990).

A9.6.3 *QSAR modelling issues*

A9.6.3.1   Choosing an appropriate QSAR implies that the model will yield a reliable prediction for the toxicity or biological activity of an untested chemical. Generally speaking, reliability decreases with increasing complexity of chemical structure, unless a QSAR has been derived for a narrowly defined set of chemicals similar in structure to the candidate substance. QSAR models derived from narrowly defined classes of chemicals are commonly employed in the development of pharmaceuticals once a new lead compound is identified and there is a need to make minor structural modifications to optimize activity (and decrease toxicity). Overall, the objective is make estimates by interpolation rather than extrapolation.

A9.6.3.2   For example, if 96-h LC_{50} test data for fathead minnow are available for ethanol, n-butanol, n-hexanol, and n-nonanol, there is some confidence in making a prediction for this endpoint for n-propanol and n-pentanol. In contrast, there is would have less confidence in making such a prediction for methanol, which is an extrapolation, with fewer carbon atoms than any of the tested chemicals. In fact, the behaviour of the first member of such a homologous is typically the most anomalous, and should not be predicted using data from remaining members of the series. Even the toxicity of branched chain alcohols may be an unreasonable extrapolation, depending upon the endpoint in question. Such extrapolation becomes more unreliable to the extent that toxicity is related to production of metabolites for a particular endpoint, as opposed to the properties of the parent compound. Also, if toxicity is mediated by a specific receptor binding mechanism, dramatic effects may be observed with small changes in chemical structure.

A9.6.3.3   What ultimately governs the validity of such predictions is the degree to which the compounds used to derive the QSAR for a specific biological endpoint, are acting by a common molecular mechanism. In many and perhaps most cases, a QSAR does not represent such a mechanistic model, but merely a correlative one. A truly valid mechanistic model must be derived from a series of chemicals all acting by a common molecular mechanism, and fit to an equation using one or more parameters that relate directly to one or more steps of the mechanism in question. Such parameters or properties are more generally known as molecular descriptors. It is also important to keep in mind that many such molecular descriptors in common use may not have a direct physical interpretation. For a correlative model, the statistical fit of the

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data are likely to be poorer than a mechanistic one given these limitations. Mechanisms are not necessarily completely understood, but enough information may be known to provide confidence in this approach. For correlative models, the predictive reliability increases with the narrowness with which each is defined, e.g. categories of electrophiles, such as acrylates, in which the degree of reactivity may be similar and toxicity can be estimated for a "new" chemical using a model based solely on the log K_{ow} parameter.

A9.6.3.4   As an example, primary and secondary alcohols containing a double or triple bond that is conjugated with the hydroxyl function (i.e. allylic or propargylic) are more toxic than would be predicted for a QSAR for the corresponding saturated compounds. This behaviour has been ascribed to a proelectrophile mechanism involving metabolic activation by the ubiquitous enzyme alcohol dehydrogenase to the corresponding α,β-unsaturated aldehydes and ketones which can act as electrophiles via a Michael-type acceptor mechanism (Veith *et al.*, 1989). In the presence of an alcohol dehydrogenase inhibitor, these compounds behave like other alcohols and do not show excess toxicity, consistent with the mechanistic hypothesis.

A9.6.3.5   The situation quickly becomes more complex once one goes beyond such a homologous series of compounds. Consider, for example, simple benzene derivatives. A series of chlorobenzenes may be viewed as similar to a homologous series. Not much difference is likely in the toxicities of the three isomeric dichlorobenzenes, so that a QSAR for chlorobenzenes based upon test data for one of these isomers is likely to be adequate. What about the substitution of other functional groups on benzene ring? Unlike an aliphatic alcohol, addition of a hydroxyl functionality to a benzene ring produces a phenol which is no longer neutral, but an ionizable acidic compound, due to the resonance stabilization of the resulting negative charge. For this reason, phenol does not act as a true narcotic agent. With the addition of electron withdrawing substituents to phenol (e.g. chlorine atoms), there is a shift to these compounds acting as uncouplers of oxidative phosphorylation (e.g. the herbicide dinoseb). Substitution of an aldehyde group leads to increased toxicity via an electrophile mechanism for such compounds react with amino groups, such as the lysine ε-amino group to produce a Schiff Base adduct. Similarly, a benzylic chloride acts as an electrophile to form covalent abducts with sulfhydryl groups. In tackling a prediction for an untested compound, the chemical reactivity of these and many other functional groups and their interaction with one another should be carefully studied, and attempts made to document these from the chemical literature (Lipnick, 1991b).

A9.6.3.6   Given these limitations in using QSARs for making predictions, it is best employed as a means of establishing testing priorities, rather than as a means of substituting for testing, unless some mechanistic information is available on the untested compound itself. In fact, the inability to make a prediction along with known environmental release and exposure may in itself be adequate to trigger testing or the development of a new QSAR for a class of chemicals for which such decisions are needed. A QSAR model can be derived by statistical analysis, e.g. regression analysis, from such a data set. The most commonly employed molecular descriptor, log K_{ow}, may be tried as a first attempt.

A9.6.3.7   By contrast, derivation of a mechanism based QSAR model requires an understanding or working hypothesis of molecular mechanism and what parameter or parameters would appropriately model these actions. It is important to keep in mind that this is different from a hypothesis regarding mode of action, which relates to biological/physiological response, but not molecular mechanism.

A9.6.4   *Use of QSARs in aquatic classification*

A9.6.4.1   The following inherent properties of substances are relevant for classification purposes concerning the aquatic environment:

        (a)   partition coefficient n-octanol-water log K_{ow};

        (b)   bioconcentration factor BCF;

        (c)   degradability - abiotic and biodegradation;

        (d)   acute aquatic toxicity for fish, daphnia and algae;

        (e)   prolonged toxicity for fish and daphnia.

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A9.6.4.2   Test data always take precedence over QSAR predications, providing the test data are valid, with QSARs used for filling data gaps for purposes of classification. Since the available QSARs are of varying reliability and application range, different restrictions apply for the prediction of each of these endpoints. Nevertheless, if a tested compound belongs to a chemical class or structure type (see above) for which there is some confidence in the predictive utility of the QSAR model, it is worthwhile to compare this prediction with the experimental data, as it is not unusual to use this approach to detect some of the experimental artefacts (volatilization, insufficient test duration to achieve equilibrium, and water solubility cut-off) in the measured data, which would mostly result in classifying substances as lower than actual toxicity.

A9.6.4.3   When two or more QSARs are applicable or appear to be applicable, it is useful to compare the predictions of these various models in the same way that predicted data should be compared with measured (as discussed above). If there is no discrepancy between these models, the result provides encouragement of the validity of the predictions. Of course, it may also mean that the models were all developed using data on similar compounds and statistical methods. On the other hand, if the predictions are quite different, this result needs to be examined further. There is always the possibility that none of the models used provides a valid prediction. As a first step, the structures and properties of the chemicals used to derive each of the predictive models should be examined to determine if any models are based upon chemicals similar in both of these respects to the one for which a prediction is needed. If one data set contains such an appropriate analogue used to derive the model, the measured value in the database for that compound vs model prediction should be tested. If the results fit well with the overall model, it is likely the most reliable one to use. Likewise, if none of the models contain test data for such an analogue, testing of the chemical in question is recommended.

A9.6.4.4   The U.S. EPA has recently posted a draft document on its website "Development of Chemical Categories in the HPV Challenge Program," that proposes the use of chemical categories to "... voluntarily compile a Screening Information Data Set (SIDS) on all chemicals on the US HPV list ... [to provide] basic screening data needed for an initial assessment of the physicochemical properties, environmental fate, and human and environmental effects of chemicals" (US EPA, 1999). This list consists of "...about 2,800 HPV chemicals which were reported for the Toxic Substances Control Act's 1990 Inventory Update Rule (IUR)".

A9.6.4.5   One approach being proposed "...where this is scientifically justifiable ... is to consider closely related chemicals as a group, or category, rather than test them as individual chemicals. In the category approach, not every chemical needs to be tested for every SIDS endpoint". Such limited testing could be justified providing that the "...final data set must allow one to assess the untested endpoints, ideally by *interpolation* [emphasis added here] between and among the category members." The process for defining such categories and in the development of such data are described in the proposal.

A9.6.4.6   A second potentially less data intensive approach being considered (US EPA, 2000a) is "... applying SAR principles to a single chemical that is closely related to one or more better characterized chemicals ("analogs")." A third approach proposed consists of using "... a combination of the analogue and category approaches ... [for] individual chemicals ... [similar to that] used in ECOSAR (US EPA, 2000b), a SAR-based computer program that generates ecotoxicity values. ". The document also details the history of the use of SARs within the U.S. EPA new chemicals program, and how to go about collecting and analysing data for the sake of such SAR approaches.

A9.6.4.7   The Nordic Council of Ministers issued a report (Pederson *et al.*, 1995) entitled "Environmental Hazard Classification," that includes information on data collection and interpretation, as well as a section (5.2.8) entitled "QSAR estimates of water solubility and acute aquatic toxicity". This section also discusses the estimation of physicochemical properties, including log K_{ow}. For the sake of classification purposes, estimation methods are recommended for prediction of "minimum acute aquatic toxicity," for "...neutral, organic, non-reactive and non-ionizable compounds such as alcohols, ketones, ethers, alkyl, and aryl halides, and can also be used for aromatic hydrocarbons, halogenated aromatic and aliphatic hydrocarbons as well as sulphides and disulphides," as cited in an earlier OECD Guidance

Document (OECD, 1995). The Nordic document also includes diskettes for a computerized application of some of these methods.

A9.6.4.8   The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) has published a report entitled "QSARs in the Assessment of the Environmental Fate and Effects of Chemicals," which describes the use of QSARs to "...check the validity of data or to fill data gaps for priority setting, risk assessment and classification" (ECETOC, 1998). QSARs are described for predicting environmental fate and aquatic toxicity. The report notes that "a consistent dataset for [an endpoint] covered ... for a well defined scope of chemical structures ("domain") [is needed] ... from which a training set is developed. The document also discusses the advantage of mechanism based models, the use of statistical analysis in the development of QSARs, and how to assess "outliers".

A9.6.4.9   *Octanol-water-partition coefficient (K_{ow})*

A9.6.4.9.1   Computerized methods such as CLOGP (US EPA, 1999), LOGKOW (US EPA, 2000a) and SPARC (US EPA, 2000b) are available to calculate log K_{ow} directly from chemical structure. CLOGP and LOGKOW are based upon the addition of group contributions, while SPARC is based upon a more fundamental chemical structure algorithm. Caution should be used in using calculated values for compounds that can undergo hydrolysis in water or some other reaction, since these transformations need to be considered in the interpretation of aquatic toxicity test data for such reactive chemicals. Only SPARC can be employed in a general way for inorganic or organometallic compounds. Special methods are needed in making estimates of log K_{ow} or aquatic toxicity for surface-active compounds, chelating compounds, and mixtures.

A9.6.4.9.2   Values of log K_{ow} can be calculated for pentachlorophenol and similar compounds, both for the ionized and unionized (neutral) forms. These values can potentially be calculated for certain reactive molecules (e.g. benzotrichloride), but the reactivity and subsequent hydrolysis also need to be considered. Also, for such ionizable phenols, pKa is a second parameter. Specific models can be used to calculate log K_{ow} values for organometallic compounds, but they need to be applied with caution since some of these compounds really exist in the form of ion pairs in water.

A9.6.4.9.3   For compounds of extremely high lipophilicity, measurements up to about 6 to 6.5 can be made by shake flask, and can be extended up to about log K_{ow} of 8 using the slow stirring approach (Bruijn *et al.*, 1989). Calculations are considered useful even in extrapolating beyond what can be measured by either of these methods. Of course, it should be kept in mind that if the QSAR models for toxicity, etc. are based on chemicals with lower log K_{ow} values, the prediction itself will also be an extrapolation; in fact, it is known that in the case of bioconcentration, the relationship with log K_{ow} becomes non-linear at higher values. For compounds with low log K_{ow} values, the group contribution can also be applied, but this is not very useful for hazard purposes since for such substances, particularly with negative log K_{ow} values, little if any partitioning can take place into lipophilic sites and as Overton reported, these substances produce toxicity through osmotic effects (Lipnick, 1986).

A9.6.4.10   *Bioconcentration factor BCF*

A9.6.4.10.1   If experimentally determined BCF values are available, these values should be used for classification. Bioconcentration measurements must be performed using pure samples at test concentrations within water solubility, and for an adequate test duration to achieve steady state equilibrium between the aqueous concentration and that in the fish tissue. Moreover, with bioconcentration tests of extended duration, the correlation with log K_{ow} levels off and ultimately decreases. Under environmental conditions, bioconcentration of highly lipophilic chemicals takes place by a combination of uptake from food and water, with the switch to food taking place at log $K_{ow} \approx 6$. Otherwise log K_{ow} values can be used with a QSAR model as a predictor of the bioaccumulation potential of organic compounds. Deviations from these QSARs tend to reflect differences in the extent to which the chemicals undergo metabolism in the fish. Thus, some chemicals, such as phthalate, can bioconcentrate significantly less than predicted for this reason. Also, caution should be applied in comparing predicted BCF values with those using radiolabeled compounds,

where the tissue concentration thus detected may represent a mix of parent compound and metabolites or even covalently bound parent or metabolite.

A9.6.4.10.2   Experimental log K_{ow} values are to be used preferentially. However, older shake flask values above 5.5 are not reliable and in many cases it is better to use some average of calculated values or to have these remeasured using the slow stirring method (Bruijn *et al.*, 1989). If there is reasonable doubt about the accuracy of the measured data, calculated log K_{ow} values shall be used.

A9.6.4.11   *Degradability - abiotic and biodegradation*

QSARs for abiotic degradation in water phases are narrowly defined linear free energy relationships (LFERs) for specific classes of chemicals and mechanisms. For example, such LFERs are available for hydrolysis of benzylic chlorides with various substituents on the aromatic ring. Such narrowly defined LFER models tend to be very reliable if the needed parameters are available for the Substituent(s) in question. Photo degradation, i.e. reaction with UV produced reactive species, may be extrapolated from estimates for the air compartment. While these abiotic processes do not usually result in complete degradation of organic compounds, they are frequently significant starting points, and may be rate limiting. QSARs for calculating biodegradability are either compound specific (OECD, 1995) or group contribution models like the BIODEG program (Hansch and Leo, 1995; Meylan and Howard 1995; Hilal *et al.*, 1994; Howard *et al.*, 1992; Boethling *et al.*, 1994; Howard and Meylan 1992; Loonen *et al.*, 1999). While validated compound class specific models are very limited in their application range, the application range of group contribution models is potentially much broader, but limited to compounds containing the model substructures. Validation studies have suggested that the biodegradability predictions by currently available group contribution models may be used for prediction of "not ready biodegradability" (Pedersen *et al.*, 1995; Langenberg *et al.*, 1996; USEPA, 1993) – and thus in relation to aquatic hazard classification "not rapid degradability."

A9.6.4.12   *Acute aquatic toxicity for fish, daphnia and algae*

The acute aquatic toxicity of non-reactive, non-electrolyte organic chemicals (baseline toxicity) can be predicted from their log K_{ow} value with a quite high level of confidence, provided the presence of electrophile, proelectrophile, or special mechanism functional groups (see above) were not detected. Problems remain for such specific toxicants, for which the appropriate QSAR has to be selected in a prospective manner. Since straightforward criteria for the identification of the relevant modes of action are still lacking, empirical expert judgement needs to be applied for selecting a suitable model. Thus, if an inappropriate QSAR is employed, the predictions may be in error by several orders of magnitude, and in the case of baseline toxicity, will be predicted less toxic, rather than more.

A9.6.4.13   *Prolonged toxicity for fish and Daphnia*

Calculated values for chronic toxicity to fish and Daphnia should not be used to overrule classification based on experimental acute toxicity data. Only a few validated models are available for calculating prolonged toxicity for fish and Daphnia. These models are based solely on log K_{ow} correlations and are limited in their application to non-reactive, non-electrolyte organic compounds, and are not suitable for chemicals with specific modes of action under prolonged exposure conditions. The reliable estimation of chronic toxicity values depends on the correct discrimination between non-specific and specific chronic toxicity mechanisms; otherwise, the predicted toxicity can be wrong by orders of magnitude. It should be noted that although for many compounds, excess toxicity[4] in a chronic test correlates with excess toxicity in an acute test, this is not always the case.

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[4]   *Excess toxicity, T_e = (Predicted baseline toxicity)/Observed toxicity.*

A9.7   **Classification of metals and metal compounds**

A9.7.1   *Introduction*

A9.7.1.1   The harmonized system for classifying chemical substances is a hazard-based system, and the basis of the identification of hazard is the aquatic toxicity of the substances, and information on the degradation and bioaccumulation behaviour (OECD 1998). Since this document deals only with the hazards associated with a given substance when the substance is dissolved in the water column, exposure from this source is limited by the solubility of the substance in water and bioavailability of the substance in species in the aquatic environment. Thus, the hazard classification schemes for metals and metal compounds are limited to the hazards posed by metals and metal compounds when they are available (i.e. exist as dissolved metal ions, for example, as M^+ when present as $M-NO_3$), and do not take into account exposures to metals and metal compounds that are not dissolved in the water column but may still be bioavailable, such as metals in foods. This section does not take into account the non-metallic ion (e.g. CN-) of metal compounds which may be toxic or which may be organic and may pose bioaccumulation or persistence hazards. For such metal compounds the hazards of the non-metallic ions must also be considered.

A9.7.1.2   The level of the metal ion which may be present in solution following the addition of the metal and/or its compounds, will largely be determined by two processes: the extent to which it can be dissolved, i.e. its water solubility, and the extent to which it can react with the media to transform to water soluble forms. The rate and extent at which this latter process, known as "transformation" for the purposes of this guidance, takes place can vary extensively between different compounds and the metal itself, and is an important factor in determining the appropriate hazard class. Where data on transformation are available, they should be taken into account in determining the classification. The Protocol for determining this rate is available in Annex 10.

A9.7.1.3   Generally speaking, the rate at which a substance dissolves is not considered relevant to the determination of its intrinsic toxicity. However, for metals and many poorly soluble inorganic metal compounds, the difficulties in achieving dissolution through normal solubilization techniques is so severe that the two processes of solubilization and transformation become indistinguishable. Thus, where the compound is sufficiently poorly soluble that the levels dissolved following normal attempts at solubilization do not exceed the available $L(E)C_{50}$, it is the rate and extent of transformation, which must be considered. The transformation will be affected by a number of factors, not least of which will be the properties of the media with respect to pH, water hardness, temperature etc. In addition to these properties, other factors such as the size and specific surface area of the particles which have been tested, the length of time over which exposure to the media takes place and, of course the mass or surface area loading of the substance in the media will all play a part in determining the level of dissolved metal ions in the water. Transformation data can generally, therefore, only be considered as reliable for the purposes of classification if conducted according to the standard Protocol in Annex 10.

A9.7.1.4   This Protocol aims at standardizing the principal variables such that the level of dissolved ion can be directly related to the loading of the substance added. It is this loading level which yields the level of metal ion equivalent to the available $L(E)C_{50}$ that can then be used to determine the hazard category appropriate for classification. The testing methodology is detailed in Annex 10. The strategy to be adopted in using the data from the testing protocol, and the data requirements needed to make that strategy work, will be described.

A9.7.1.5   In considering the classification of metals and metal compounds, both readily and poorly soluble, recognition has to be paid to a number of factors. As defined in Chapter 4.1, the term "degradation" refers to the decomposition of organic molecules. For inorganic compounds and metals, clearly the concept of degradability, as it has been considered and used for organic substances, has limited or no meaning. Rather, the substance may be transformed by normal environmental processes to either increase or decrease the bioavailability of the toxic species. Equally, the log K_{ow} cannot be considered as a measure of the potential to accumulate. Nevertheless, the concepts that a substance, or a toxic metabolite/reaction product may not be rapidly lost from the environment and/or may bioaccumulate are as applicable to metals and metal compounds as they are to organic substances.

A9.7.1.6   Speciation of the soluble form can be affected by pH, water hardness and other variables, and may yield particular forms of the metal ion which are more or less toxic. In addition, metal ions could be made non-available from the water column by a number of processes (e.g. mineralization and partitioning). Sometimes these processes can be sufficiently rapid to be analogous to degradation in assessing chronic classification. However, partitioning of the metal ion from the water column to other environmental media does not necessarily mean that it is no longer bioavailable, nor does it mean that the metal has been made permanently unavailable.

A9.7.1.7   Information pertaining to the extent of the partitioning of a metal ion from the water column, or the extent to which a metal has been or can be converted to a form that is less toxic or non-toxic is frequently not available over a sufficiently wide range of environmentally relevant conditions, and thus, a number of assumptions will need to be made as an aid in classification. These assumptions may be modified if available data show otherwise. In the first instance it should be assumed that the metal ions, once in the water, are not rapidly partitioned from the water column and thus these compounds do not meet the criteria. Underlying this is the assumption that, although speciation can occur, the species will remain available under environmentally relevant conditions. This may not always be the case, as described above, and any evidence available that would suggest changes to the bioavailability over the course of 28 days, should be carefully examined. The bioaccumulation of metals and inorganic metal compounds is a complex process and bioaccumulation data should be used with care. The application of bioaccumulation criteria will need to be considered on a case-by-case basis taking due account of all the available data.

A9.7.1.8   A further assumption that can be made, which represents a cautious approach, is that, in the absence of any solubility data for a particular metal compound, either measured or calculated, the substance will be sufficiently soluble to cause toxicity at the level of the $L(E)C_{50}$, and thus may be classified in the same way as other soluble salts. Again, this is clearly not always the case, and it may be wise to generate appropriate solubility data.

A9.7.1.9   This section deals with metals and metal compounds. Within the context of this Guidance Document, metals and metal compounds are characterized as follows, and therefore, organo-metals are outside the scope of this section:

(a)   metals, M^0, in their elemental state are not soluble in water but may transform to yield the available form. This means that a metal in the elemental state may react with water or a dilute aqueous electrolyte to form soluble cationic or anionic products, and in the process the metal will oxidize, or transform, from the neutral or zero oxidation state to a higher one;

(b)   in a simple metal compound, such as an oxide or sulphide, the metal already exists in the oxidized state, so that further metal oxidation is unlikely to occur when the compound is introduced into an aqueous medium.

However, while oxidization may not change, interaction with the media may yield more soluble forms. A sparingly soluble metal compound can be considered as one for which a solubility product can be calculated, and which will yield a small amount of the available form by dissolution. However, it should be recognized that the final solution concentration may be influenced by a number of factors, including the solubility product of some metal compounds precipitated during the transformation/dissolution test, e.g. aluminium hydroxide.

A9.7.2   *Application of aquatic toxicity data and solubility data for classification*

A9.7.2.1   *Interpretation of aquatic toxicity data*

A9.7.2.1.1   Aquatic toxicity studies carried out according to a recognized protocol should normally be acceptable as valid for the purposes of classification. Section A9.3 should also be consulted for generic issues that are common to assessing any aquatic toxicity data point for the purposes of classification.

A9.7.2.1.2   Metal complexation and speciation

A9.7.2.1.2.1   The toxicity of a particular metal in solution, appears to depend primarily on (but is not strictly limited to) the level of dissolved free metal ions. Abiotic factors including alkalinity, ionic strength and pH can influence the toxicity of metals in two ways: (i) by influencing the chemical speciation of the metal in water (and hence affecting the availability) and (ii) by influencing the uptake and binding of available metal by biological tissues.

A9.7.2.1.2.2   Where speciation is important, it may be possible to model the concentrations of the different forms of the metal, including those that are likely to cause toxicity. Analysis methods for quantifying exposure concentrations, which are capable of distinguishing between the complexed and uncomplexed fractions of a test substance, may not always be available or economic.

A9.7.2.1.2.3   Complexation of metals to organic and inorganic ligands in test media and natural environments can be estimated from metal speciation models. Speciation models for metals, including pH, hardness, DOC, and inorganic substances such as MINTEQ (Brown and Allison, 1987), WHAM (Tipping, 1994) and CHESS (Santore and Driscoll, 1995) can be used to calculate the uncomplexed and complexed fractions of the metal ions. Alternatively, the Biotic Ligand Model (BLM), allows for the calculation of the concentration of metal ion responsible for the toxic effect at the level of the organism. The BLM model has at present only been validated for a limited number of metals, organisms, and end-points (Santore and Di Toro, 1999). The models and formula used for the characterization of metal complexation in the media should always be clearly reported, allowing for their translation back to natural environments (OECD, 2000).

A9.7.2.2   *Interpretation of solubility data*

A9.7.2.2.1   When considering the available data on solubility, their validity and applicability to the identification of the hazard of metal compounds should be assessed. In particular, a knowledge of the pH at which the data were generated should be known.

A9.7.2.2.2   Assessment of existing data

Existing data will be in one of three forms. For some well-studied metals, there will be solubility products and/or solubility data for the various inorganic metal compounds. It is also possible that the pH relationship of the solubility will be known. However, for many metals or metal compounds, it is probable that the available information will be descriptive only, e.g. poorly soluble. Unfortunately there appears to be very little (consistent) guidance about the solubility ranges for such descriptive terms. Where these are the only information available it is probable that solubility data will need to be generated using the Transformation/Dissolution Protocol (Annex 10).

A9.7.2.2.3   Screening test for assessing solubility of metal compounds

In the absence of solubility data, a simple "Screening Test" for assessing solubility, based on the high rate of loading for 24 h, can be used for metal compounds as described in the Transformation/Dissolution Protocol (Annex 10). The function of the screening test is to identify those metal compounds which undergo either dissolution or rapid transformation such that they are indistinguishable from soluble forms and hence may be classified based on the dissolved ion concentration. Where data are available from the screening test detailed in the Transformation/Dissolution Protocol, the maximum solubility obtained over the tested pH range should be used. Where data are not available over the full pH range, a check should be made that this maximum solubility has been achieved by reference to suitable thermodynamic speciation models or other suitable methods (see A9.7.2.1.2.3). It should be noted that this test is only intended to be used for metal compounds.

A9.7.2.2.4   Full test for assessing solubility of metals and metal compounds

The first step in this part of the study is, as with the screening test, an assessment of the pH(s) at which the study should be conducted. Normally, the Full Test should have been carried out at the pH that maximizes the concentration of dissolved metal ions in solution. In such cases, the pH may be chosen following the same guidance as given for the screening test.

Based on the data from the Full Test, it is possible to generate a concentration of the metal ions in solution after 7 days for each of the three loadings (i.e. 1 mg/l as "low", 10 mg/l as "medium" and 100 mg/l as "high") used in the test. If the purpose of the test is to assess the long-term hazard of the substance, then the test at the low loading may be extended to 28 days, at an appropriate pH.

A9.7.2.3   *Comparison of aquatic toxicity data and solubility data*

A decision whether or not the substance be classified will be made by comparing aquatic toxicity data and solubility data. If the $L(E)C_{50}$ is exceeded, irrespective of whether the toxicity and dissolution data are at the same pH and if this is the only data available then the substance should be classified. If other solubility data are available to show that the dissolution concentration would not exceed the $L(E)C_{50}$ across the entire pH range then the substance should not be classified on its soluble form. This may involve the use of additional data either from ecotoxicological testing or from applicable bioavailability-effect models.

A9.7.3   *Assessment of environmental transformation*

A9.7.3.1   Environmental transformation of one species of a metal to another species of the same does not constitute degradation as applied to organic compounds and may increase or decrease the availability and bioavailability of the toxic species. However as a result of naturally occurring geochemical processes metal ions can partition from the water column. Data on water column residence time, the processes involved at the water – sediment interface (i.e. deposition and re-mobilization) are fairly extensive, but have not been integrated into a meaningful database. Nevertheless, using the principles and assumptions discussed above in A9.7.1, it may be possible to incorporate this approach into classification.

A9.7.3.2   Such assessments are very difficult to give guidance for and will normally be addressed on a case by case approach. However, the following may be taken into account:

(a)   Changes in speciation if they are to non-available forms, however, the potential for the reverse change to occur must also be considered;

(b)   Changes to a metal compound which is considerably less soluble than that of the metal compound being considered.

Some caution is recommended, see A9.7.1.5 and A9.7.1.6.

A9.7.4   *Bioaccumulation*

A9.7.4.1   While log K_{ow} is a good predictor of BCF for certain types of organic compounds e.g. non-polar organic substances, it is of course irrelevant for inorganic substances such as inorganic metal compounds.

A9.7.4.2   The mechanisms for uptake and depuration rates of metals are very complex and variable and there is at present no general model to describe this. Instead the bioaccumulation of metals according to the classification criteria should be evaluated on a case-by-case basis using expert judgement.

A9.7.4.3   While BCFs are indicative of the potential for bioaccumulation there may be a number of complications in interpreting measured BCF values for metals and inorganic metal compounds. For some metals and inorganic metal compounds the relationship between water concentration and BCF in some

aquatic organisms is inverse, and bioconcentration data should be used with care. This is particularly relevant for metals that are biologically essential. Metals that are biologically essential are actively regulated in organisms in which the metal is essential. Since nutritional requirement of the organisms can be higher than the environmental concentration, this active regulation can result in high BCFs and an inverse relationship between BCFs and the concentration of the metal in water. When environmental concentrations are low, high BCFs may be expected as a natural consequence of metal uptake to meet nutritional requirements and in these instances can be viewed as a normal phenomenon. Additionally, if internal concentration is regulated by the organism, then measured BCFs may decline as external concentration increases. When external concentrations are so high that they exceed a threshold level or overwhelm the regulatory mechanism, this can cause harm to the organism. Also, while a metal may be essential in a particular organism, it may not be essential in other organisms. Therefore, where the metal is not essential or when the bioconcentration of an essential metal is above nutritional levels special consideration should be given to the potential for bioconcentration and environmental concern.

A9.7.5 *Application of classification criteria to metals and metal compounds*

A9.7.5.1 *Introduction to the classification strategy for metals and metal compounds*

A9.7.5.1.1 The schemes for the classification of metals and metal compounds are described below and summarized diagrammatically in Figure A9.7.1. There are several stages in these schemes where data are used for decision purposes. It is not the intention of the classification schemes to generate new data. In the absence of valid data, it will be necessary to use all available data and expert judgement.

In the following sections, the reference to the $L(E)C_{50}$ refers to the data point(s) that will be used to select the class for the metal or metal compound.

A9.7.5.1.2 When considering $L(E)C_{50}$ data for metal compounds, it is important to ensure that the data point to be used as the justification for the classification is expressed in the weight of the molecule of the metal compound to be classified. This is known as correcting for molecular weight. Thus while most metal data is expressed in, for example, mg/l of the metal, this value will need to be adjusted to the corresponding weight of the metal compound. Thus:

$$L(E)C_{50} \text{ metal compounds} = L(E)C_{50} \text{ of metal} \times (\text{Molecular weight of metal compound} / \text{Atomic weight of metal})$$

NOEC data may also need to be adjusted to the corresponding weight of the metal compounds.

A9.7.5.2 *Classification strategy for metals*

A9.7.5.2.1 Where the $L(E)C_{50}$ for the metal ions of concern is greater than 100 mg/l, the metals need not be considered further in the classification scheme.

A9.7.5.2.2 Where the $L(E)C_{50}$ for the metal ions of concern is ≤100 mg/l, consideration must be given to the data available on the rate and extent to which these ions can be generated from the metal. Such data, to be valid and useable should have been generated using the Transformation/Dissolution Protocol (Annex 10).

A9.7.5.2.3 Where such data are unavailable, i.e. there is no clear data of sufficient validity to show that the transformation to metal ions will not occur, the safety net classification (Chronic Category 4) should be applied since the known classifiable toxicity of these soluble forms is considered to produce sufficient concern.

A9.7.5.2.4 Where data from dissolution protocol are available, then, the results should be used to aid classification according to the following rules:

A9.7.5.2.4.1   7 day Transformation Test

If the dissolved metal ion concentration after a period of 7 days (or earlier) exceeds that of the $L(E)C_{50}$, then the default classification for the metals is replaced by the following classification:

(a)   If the dissolved metal ion concentration at the low loading rate is $\geq L(E)C_{50}$, then classify Acute Category 1. Classify also as Chronic Category 1, unless there is evidence of both rapid partitioning from the water column and no bioaccumulation;

(b)   If the dissolved metal ion concentration at the medium loading rate is $\geq L(E)C_{50}$, then classify Acute Category 2. Classify also as Chronic Category 2 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation;

(c)   If the dissolved metal ion concentration at the high loading rate is $\geq L(E)C_{50}$, then classify Acute Category 3. Classify also as Chronic Category 3 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation.

A9.7.5.2.4.2   28 day transformation test

If the process described in A9.7.5.2.4.1 results in the classification of Chronic Category 1, no further assessment is required, as the metal will be classified irrespective of any further information.

In all other cases, further data may have been generated through the dissolution/transformation test in order to show that the classification may be amended. If for substances classified Chronic Category 2, 3 or 4, the dissolved metal ion concentration at the low loading rate after a total period of 28 days is $\leq$ long-term NOECs, then the classification is removed.

A9.7.5.3   *Classification strategy for metal compounds*

A9.7.5.3.1   Where the $L(E)C_{50}$ for the metal ions of concern is > 100 mg/l, the metal compounds need not be considered further in the classification scheme.

A9.7.5.3.2   If solubility $\geq L(E)C_{50}$, classify on the basis of soluble ion.

A9.7.5.3.2.1   All metal compounds with a water solubility (either measured e.g. through 24-hour Dissolution Screening test or estimated e.g. from the solubility product) $\geq L(E)C_{50}$ of the dissolved metal ion concentration are considered as readily soluble metal compounds. Care should be exercised for compounds whose solubility is close to the acute toxicity value as the conditions under which solubility is measured could differ significantly from those of the acute toxicity test. In these cases the results of the Dissolution Screening Test are preferred.

A9.7.5.3.2.2   Readily soluble metal compounds are classified on the basis of the $L(E)C_{50}$ (corrected where necessary for molecular weight):

(a)   If the $L(E)C_{50}$ of the dissolved metal ion is $\leq$ 1 mg/l then classify Acute Category 1. Classify also as Chronic Category 1 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation;

(b)   If the $L(E)C_{50}$ of the dissolved metal ion is >1 mg/l but $\leq$ 10 mg/l then classify Acute Category 2. Classify also as Chronic Category 2 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation;

(c)   If the $L(E)C_{50}$ of the dissolved metal ion is > 10 mg/l and $\leq$ 100 mg/l then classify Acute Category 3. Classify also as Chronic Category 3 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation.

A9.7.5.3.3   *If solubility < L(E)C$_{50}$, classify default Chronic Category 4*

A9.7.5.3.3.1   In the context of the classification criteria, poorly soluble compounds of metals are defined as those with a known solubility (either measured e.g. through 24-hour Dissolution Screening test or estimated e.g. from the solubility product) less than the L(E)C$_{50}$ of the soluble metal ion. In those cases when the soluble forms of the metal of poorly soluble metal compounds have a L(E)C$_{50}$ ≤ 100 mg/l and the substance can be considered as poorly soluble the default safety net classification (Chronic Category 4) should be applied.

A9.7.5.3.3.2   7 day Transformation Test

For poorly soluble metal compounds classified with the default safety net classification further information that may be available from the 7-day transformation/dissolution test can also be used. Such data should include transformation levels at low, medium and high loading levels.

If the dissolved metal ion concentration after a period of 7 days (or earlier) exceeds that of the L(E)C$_{50}$, then the default classification for the metals is replaced by the following classification:

(a)   If the dissolved metal ion concentration at the low loading rate is ≥L(E)C$_{50}$, then classify Acute Category 1. Classify also as Chronic Category 1, unless there is evidence of both rapid partitioning from the water column and no bioaccumulation;

(b)   If the dissolved metal ion concentration at the medium loading rate is ≥ L(E)C$_{50}$, then classify Acute Category 2. Classify also as Chronic Category 2 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation;

(c)   If the dissolved metal ion concentration at the high loading rate is ≥ L(E)C$_{50}$, then classify Acute Category 3 . Classify also as Chronic Category 3 unless there is evidence of both rapid partitioning from the water column and no bioaccumulation.

A9.7.5.3.3.3   28 day Transformation test

If the process described in A9.7.5.3.3.2 results in the classification of Chronic Category 1, no further assessment is required as the metal compound will be classified irrespective of any further information.

In all other cases, further data may have been generated through the dissolution/transformation test for 28 days in order to show that the classification may be amended. If for poorly soluble metal compounds classified as Chronic Category 2, 3 or 4, the dissolved metal ion concentration at the low loading rate after a total period of 28 days is less than or equal to the long-term NOECs, then classification is removed.

A9.7.5.4   *Particle size and surface area*

A9.7.5.4.1   Particle size, or moreover surface area, is a crucial parameter in that any variation in the size or surface area tested may cause a significant change in the levels of metals ions released in a given time-window. Thus, this particle size or surface area is fixed for the purposes of the transformation test, allowing the comparative classifications to be based solely on the loading level. Normally, the classification data generated would have used the smallest particle size marketed to determine the extent of transformation. There may be cases where data generated for a particular metal powder is not considered as suitable for classification of the massive forms. For example, where it can be shown that the tested powder is structurally a different material (e.g. different crystallographic structure) and/or it has been produced by a special process and cannot be generated from the massive metal, classification of the massive can be based on testing of a more representative particle size or surface area, if such data are available. The powder may be classified separately based on the data generated on the powder. However, in normal circumstances it is not anticipated that more than two classification proposals would be made for the same metal.
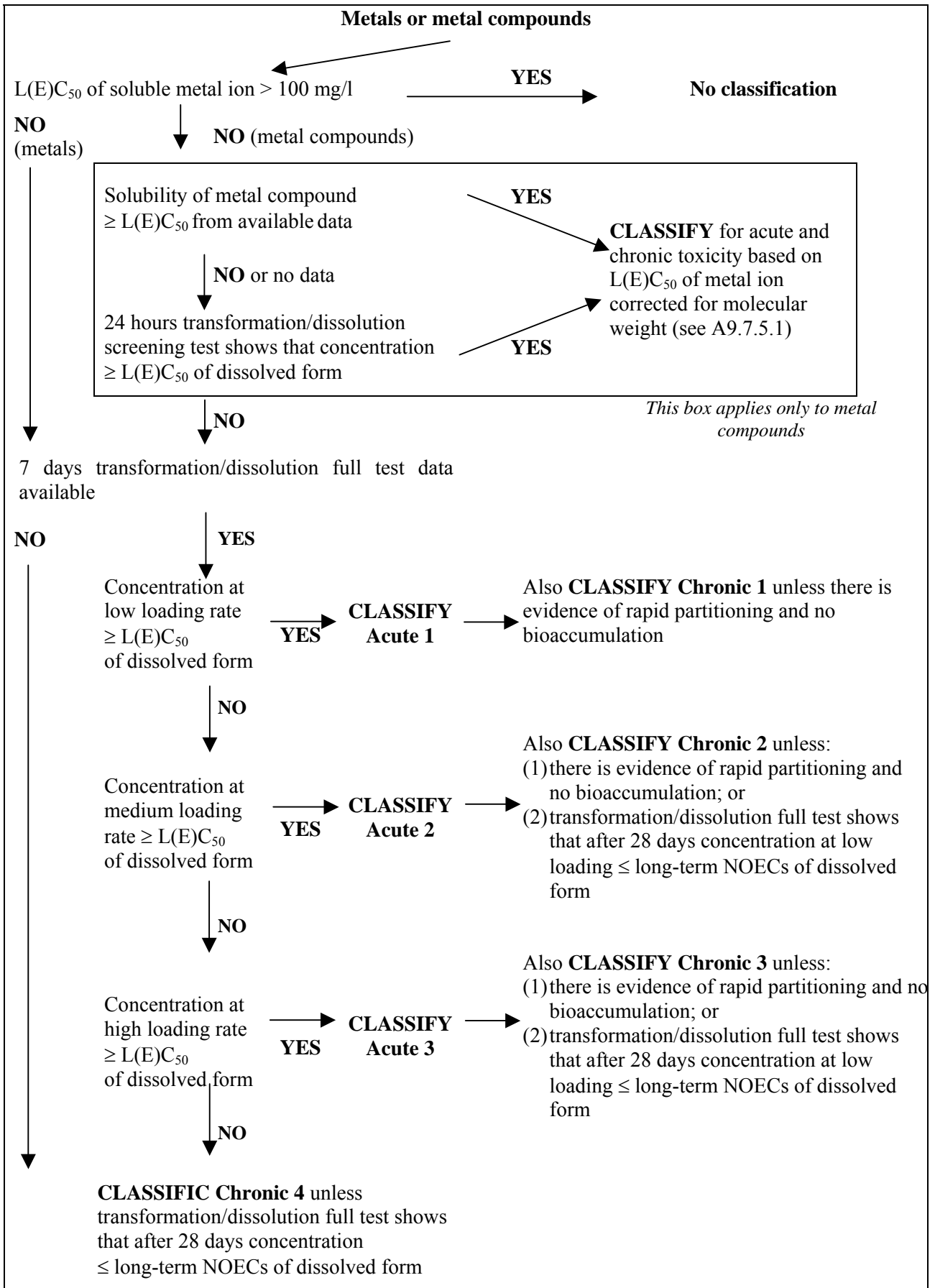
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A9.7.5.4.2   Metals with a particle size smaller than the default diameter value of 1 mm can be tested on a case-by-case basis. One example of this is where metal powders are produced by a different production technique or where the powders give rise to a higher dissolution (or reaction) rate than the massive form leading to a more stringent classification.

A9.7.5.4.3   The particle sizes tested depend on the substance being assessed and are shown in the table below:

Type	Particle size	Comments
Metal compounds	Smallest representative size sold	Never larger than 1 mm
Metals – powders	Smallest representative size sold	May need to consider different sources if yielding different crystallographic/morphologic properties
Metals – massive	1 mm	Default value may be altered if sufficient justification

A9.7.5.4.4   For some forms of metals, it may be possible, using the Transformation/Dissolution Protocol (OECD 2001), to obtain a correlation between the concentration of the metal ion after a specified time interval as a function of the surface area loadings of the forms tested. In such cases, it could then be possible to estimate the level of dissolved metal ion concentration of the metal with different particles, using the critical surface area approach as proposed by Skeaff *et. al.* (2000) (See reference in appendix VI, part 5, Metals and metal compounds). That is, from this correlation and a linkage to the appropriate toxicity data, it may be possible to determine a critical surface area of the substance that delivers the $L(E)C_{50}$ to the medium and then to convert the critical surface area to the low, medium and high mass loadings used in hazard identification. While this approach is not normally used for classification it may provide useful information for labelling and downstream decisions.

Figure A9.7.1: Classification strategy for metals and metal compounds

Metals or metal compounds

L(E)C$_{50}$ of soluble metal ion > 100 mg/l — **YES** → **No classification**

NO (metals)

NO (metal compounds) ↓

Solubility of metal compound ≥ L(E)C$_{50}$ from available data — **YES** →

CLASSIFY for acute and chronic toxicity based on L(E)C$_{50}$ of metal ion corrected for molecular weight (see A9.7.5.1)

NO or no data ↓

24 hours transformation/dissolution screening test shows that concentration ≥ L(E)C$_{50}$ of dissolved form — **YES** →

This box applies only to metal compounds

↓ **NO**

7 days transformation/dissolution full test data available

NO

↓ **YES**

Concentration at low loading rate ≥ L(E)C$_{50}$ of dissolved form — **YES** → **CLASSIFY Acute 1** → Also **CLASSIFY Chronic 1** unless there is evidence of rapid partitioning and no bioaccumulation

↓ **NO**

Concentration at medium loading rate ≥ L(E)C$_{50}$ of dissolved form — **YES** → **CLASSIFY Acute 2** → Also **CLASSIFY Chronic 2** unless:
(1) there is evidence of rapid partitioning and no bioaccumulation; or
(2) transformation/dissolution full test shows that after 28 days concentration at low loading ≤ long-term NOECs of dissolved form

↓ **NO**

Concentration at high loading rate ≥ L(E)C$_{50}$ of dissolved form — **YES** → **CLASSIFY Acute 3** → Also **CLASSIFY Chronic 3** unless:
(1) there is evidence of rapid partitioning and no bioaccumulation; or
(2) transformation/dissolution full test shows that after 28 days concentration at low loading ≤ long-term NOECs of dissolved form

↓ **NO**

CLASSIFIC Chronic 4 unless transformation/dissolution full test shows that after 28 days concentration ≤ long-term NOECs of dissolved form

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Annex 9

APPENDIX I

Determination of degradability of organic substances

1.   Organic substances may be degraded by abiotic or biotic processes or by a combination of these. A number of standard procedures or tests for determination of the degradability are available. The general principles of some of these are described below. It is by no way the intention to present a comprehensive review of degradability test methods, but only to place the methods in the context of aquatic hazard classification.

2.   Abiotic degradability

2.1   Abiotic degradation comprises chemical transformation and photochemical transformation. Usually abiotic transformations will yield other organic compounds but will not cause a full mineralization (Schwarzenbach *et al*., 1993). Chemical transformation is defined as transformation that happens without light and without the mediation of organisms whereas photochemical transformations require light.

2.2   Examples of relevant chemical transformation processes in aqueous environment are hydrolysis, nucleophilic substitution, elimination, oxidation and reduction reactions (Schwarzenbach *et al.*, 1993). Of these, hydrolysis is often considered the most important and it is the only chemical transformation process for which international test guidelines are generally available. The tests for abiotic degradation of chemicals are generally in the form of determination of transformation rates under standardized conditions.

2.3   *Hydrolysis*

2.3.1   Hydrolysis is the reaction of the nucleophiles H_2O or OH^- with a chemical where a (leaving) group of the chemical is exchanged with an OH group. Many compounds, especially acid derivatives, are susceptible to hydrolysis. Hydrolysis can both be abiotic and biotic, but in regard to testing only abiotic hydrolysis is considered. Hydrolysis can take place by different mechanisms at different pHs, neutral, acid- or base-catalysed hydrolysis, and hydrolysis rates may be very dependent on pH.

2.3.2   Currently two guidelines for evaluating abiotic hydrolysis are generally available, the OECD Test Guideline 111 Hydrolysis as a function of pH (corresponding to OPPTS 835.2110) and OPPTS 835.2130 Hydrolysis as a function of pH and temperature. In OECD Test Guideline 111, the overall hydrolysis rate at different pHs in pure buffered water is determined. The test is divided in two, a preliminary test that is performed for chemicals with unknown hydrolysis rates and a more detailed test that is performed for chemicals that are known to be hydrolytically unstable and for chemicals for which the preliminary test shows fast hydrolysis. In the preliminary test the concentration of the chemical in buffered solutions at pHs in the range normally found in the environment (pHs of 4, 7 and 9) at 50°C is measured after 5 days. If the concentration of the chemical has decreased less than 10 % it is considered hydrolytically stable, otherwise the detailed test may be performed. In the detailed test, the overall hydrolysis rate is determined at three pHs (4, 7 and 9) by measuring the concentration of the chemical as a function of time. The hydrolysis rate is determined at different temperatures so that interpolations or extrapolations to environmentally relevant temperatures can be made. The OPPTS 835.2130 test is almost identical in design to the OECD Test Guideline 111, the difference mainly being in the treatment of data.

2.3.3   It should be noted that apart from hydrolysis the hydrolysis rate constants determined by the tests include all other abiotic transformations that may occur without light under the given test conditions. Good agreement has been found between hydrolysis rates in natural and in pure waters (OPPTS 835.2110).

2.4   *Photolysis*

2.4.1   At present, there is no OECD guideline on aqueous photodegradation, but a guidance document, concerning aquatic direct photolysis, is available (OECD, 1997). The Guidance Document is supposed to form the basis for a scheduled guideline. According to the definitions set out in this Guidance Document, phototransformation of compounds in water can be in the form of primary or secondary phototransformation, where the primary phototransformation (photolysis) can be divided further into direct and indirect photolysis. Direct phototransformation (photolysis) is the case where the chemical absorbs light and as a direct result hereof undergoes transformation. Indirect phototransformation is the case where other excited species transfer energy, electrons or H-atoms to the chemical and thereby induces a transformation (sensitized photolysis). Secondary phototransformation is the case where chemical reactions occur between the chemical and reactive short lived species like hydroxy radicals, peroxy radicals or singlet oxygen that are formed in the presence of light by reactions of excited species like excited humic or fulvic acids or nitrate.

2.4.2   The only currently available guidelines on phototransformation of chemicals in water are therefore OPPTS 835.2210 *Direct photolysis rate in water by sunlight* and OPPTS 835.5270 *Indirect photolysis screening test*. The OPPTS 835.2210 test uses a tiered approach. In Tier 1 the maximum direct photolysis rate constant (minimum half-life) is calculated from a measured molar absorptivity. In Tier 2 there are two phases. In Phase 1 the chemical is photolysed with sunlight and an approximate rate constant is obtained. In Phase 2, a more accurate rate constant is determined by using an actinometer that quantifies the intensity of the light that the chemical has actually been exposed to. From the parameters measured, the actual direct photodegradation rate at different temperatures and for different latitudes can be calculated. This degradation rate will only apply to the uppermost layer of a water body, e.g. the first 50 cm or less and only when the water is pure and air saturated which may clearly not be the case in environment. However, the results can be extended over other environmental conditions by the use of a computer programme incorporating attenuation in natural waters and other relevant factors.

2.4.3   The OPPTS 835.5270 screening test concerns indirect photolysis of chemicals in waters that contain humic substances. The principle of the test is that in natural waters exposed to natural sunlight a measured phototransformation rate will include both direct and indirect phototransformation, whereas only direct phototransformation will take place in pure water. Therefore, the difference between the direct photodegradation rate in pure water and the total photodegradation in natural water is the sum of indirect photolysis and secondary photodegradation according to the definitions set out in the Annex 9 Guidance Document. In the practical application of the test, commercial humic substances are used to make up a synthetic humic water, which mimics a natural water. It should be noted that the indirect phototransformation rate determined is only valid for the season and latitude for which it is determined and it is not possible to transfer the results to other latitudes and seasons.

3.   **Biotic degradability**

3.1   Only a brief overview of the test methods is given below. For more information, the comprehensive OECD Detailed Review Paper on Biodegradability Testing (OECD, 1995) should be consulted.

3.2   *Ready biodegradability*

3.2.1   Standard tests for determination of the ready biodegradability of organic substances are developed by a number of organisations including OECD (OECD Test Guidelines 301A-F), EU (C.4 tests), OPPTS (835.3110) and ISO (9408, 9439, 10707).

3.2.2   The ready biodegradability tests are stringent tests, which provide limited opportunity for biodegradation and acclimatization to occur. The basic test conditions ensuring these specifications are:

      (a)   high concentration of test substance (2-100 mg/l);

      (b)   the test substance is the sole carbon and energy source;

(c)   low to medium concentration of inoculum (10^4-10^8 cells/mL);

(d)   no pre-adaptation of inoculum is allowed;

(e)   28 days test period with a 10-days time window (except for the MITI I method (OECD Test Guideline 301C)) for degradation to take place;

(f)   test temperature < 25 °C; and

(g)   pass levels of 70% (DOC removal) or 60% (O_2 demand or CO_2 evolution) demonstrating complete mineralization (as the remaining carbon of the test substance is assumed to be built into the growing biomass).

3.2.3   It is assumed that a positive result in one of the ready biodegradability tests demonstrates that the substance will degrade rapidly in the environment (OECD Test Guidelines).

3.2.4   Also the traditional BOD_5 tests (e.g. the EU C.5 test) may demonstrate whether a substance is readily biodegradable. In this test, the relative biochemical oxygen demand in a period of 5 days is compared to the theoretical oxygen demand (ThOD) or, when this is not available, the chemical oxygen demand (COD). The test is completed within five days and consequently, the pass level defined in the proposed hazard classification criteria at 50% is lower than in the ready biodegradability tests.

3.2.5   The screening test for biodegradability in seawater (OECD Test Guideline 306) may be seen as seawater parallel to the ready biodegradability tests. Substances that reach the pass level in OECD Test Guideline 306 (i.e. >70% DOC removal or >60 theoretical oxygen demand) may be regarded as readily biodegradable, since the degradation potential is normally lower in seawater than in the freshwater degradation tests.

3.3   *Inherent biodegradability*

3.3.1   Tests for inherent biodegradability are designed to assess whether a substance has any potential for biodegradation. Examples of such tests are the OECD Test Guidelines 302A-C tests, the EU C.9 and C.12 tests, and the ASTM E 1625-94 test.

3.3.2   The basic test conditions favouring an assessment of the inherent biodegradation potential are:

(a)   a prolonged exposure of the test substance to the inoculum allowing adaptation within the test period;

(b)   a high concentration of micro-organisms;

(c)   a favourable substance/biomass ratio.

3.3.3   A positive result in an inherent test indicates that the test substance will not persist indefinitely in the environment, however a rapid and complete biodegradation can not be assumed. A result demonstrating more than 70% mineralization indicates a potential for ultimate biodegradation, a degradation of more than 20% indicates inherent, primary biodegradation, and a result of less than 20% indicates that the substance is persistent. Thus, a negative result means that non-biodegradability (persistence) should be assumed (OECD Test Guidelines).

3.3.4   In many inherent biodegradability tests only the disappearance of the test substance is measured. Such a result only demonstrates a primary biodegradability and not a total mineralization. Thus, more or less persistent degradation products may have been formed. Primary biodegradation of a substance is no indication of ultimate degradability in the environment.

3.3.5   The OECD inherent biodegradation tests are very different in their approach and especially, the MITI II test (OECD Test Guideline 302C) employs a concentration of inoculum that is only three times

higher than in the corresponding MITI I ready biodegradability test (OECD Test Guideline 301C). Also the Zahn-Wellens test (OECD Test Guideline 302B) is a relatively "weak" inherent test. However, although the degradation potential in these tests is not very much stronger than in the ready biodegradability tests, the results can not be extrapolated to conditions in the ready biodegradability tests and in the aquatic environment.

3.4 *Aquatic simulation tests*

3.4.1   A simulation test attempts to simulate biodegradation in a specific aquatic environment. As examples of a standard test for simulation of degradation in the aquatic environment may be mentioned the ISO/DS14592 Shake flask batch test with surface water or surface water/sediment suspensions (Nyholm and Toräng, 1999), the ASTM E 1279-89(95) test on biodegradation by a shake-flask die-away method and the similar OPPTS 835.3170 test. Such test methods are often referred to as river die-away tests.

3.4.2   The features of the tests that ensure simulation of the conditions in the aquatic environment are:

(a)   use of a natural water (and sediment) sample as inoculum; and

(b)   low concentration of test substance (1-100 µg/l) ensuring first-order degradation kinetics.

3.4.3   The use of a radiolabelled test compound is recommended as this facilitates the determination of the ultimate degradation. If only the removal of the test substance by chemical analysis is determined, only the primary degradability is determined. From observation of the degradation kinetics, the rate constant for the degradation can be derived. Due to the low concentration of the test substance, first-order degradation kinetics are assumed to prevail.

3.4.4   The test may also be conducted with natural sediment simulating the conditions in the sediment compartment. Moreover, by sterilizing the samples, the abiotic degradation under the test conditions can be determined.

3.5 *STP simulation tests*

Tests are also available for simulating the degradability in a sewage treatment plant (STP), e.g. the OECD Test Guideline 303A Coupled Unit test, ISO 11733 Activated sludge simulation test, and the EU C.10 test. Recently, a new simulation test employing low concentrations of organic pollutants has been proposed (Nyholm et. al., 1996).

3.6 *Anaerobic degradability*

3.6.1   Test methods for anaerobic biodegradability determine the intrinsic potential of the test substance to undergo biodegradation under anaerobic conditions. Examples of such tests are the ISO 11734:1995(E) test, the ASTM E 1196-92 test and the OPPTS 835.3400 test.

3.6.2   The potential for anaerobic degradation is determined during a period of up to eight weeks and with the test conditions indicated below:

(a)   performance of the test in sealed vessels in the absence of O_2 (initially in a pure N_2 atmosphere);

(b)   use of digested sludge;

(c)   a test temperature of 35 °C; and

(d)   determination of head-space gas pressure (CO_2 and CH_4 formation).

3.6.3   The ultimate degradation is determined by determining the gas production. However, also primary degradation may be determined by measuring the remaining parent substance.

3.7   *Degradation in soil and sediment*

3.7.1   Many chemical substances end up in the soil or sediment compartments and an assessment of their degradability in these environments may therefore be of importance. Among standard methods may be mentioned the OECD Test Guideline 304A test on inherent biodegradability in soil, which corresponds to the OPPTS 835.3300 test.

3.7.2   The special test characteristics ensuring the determination of the inherent degradability in soil are:

    (a)   natural soil samples are used without additional inoculation;

    (b)   radiolabelled test substance is used; and

    (c)   evolution of radiolabelled CO_2 is determined.

3.7.3   A standard method for determining the biodegradation in sediment is the OPPTS 835.3180 Sediment/water microcosm biodegradation test. Microcosms containing sediment and water are collected from test sites and test compounds are introduced into the system. Disappearance of the parent compound (i.e. primary biodegradation) and, if feasible, appearance of metabolites or measurements of ultimate biodegradation may be made.

3.7.4   Currently, two new OECD guidelines are being drafted on aerobic and anaerobic transformation in soil (OECD Test Guideline, 1999a) and in aquatic sediment systems (OECD Test Guideline 1999b), respectively. The experiments are performed to determine the rate of transformation of the test substance and the nature and rates of formation and decline of transformation products under environmentally realistic conditions including a realistic concentration of the test substance. Either complete mineralization or primary degradability may be determined depending on the analytical method employed for determining the transformation of the test substance.

3.8   *Methods for estimating biodegradability*

3.8.1   In recent years, possibilities for estimating environmental properties of chemical substances have been developed and, among these, also methods for predicting the biodegradability potential of organic substances (e.g. the Syracuse Research Corporation's Biodegradability Probability Program, BIOWIN). Reviews of methods have been performed by OECD (1993) and by Langenberg *et al.* (1996). They show that group contribution methods seem to be the most successful methods. Of these, the Biodegradation Probability Program (BIOWIN) seems to have the broadest application. It gives a qualitative estimate of the probability of slow or fast biodegradation in the presence of a mixed population of environmental micro-organisms. The applicability of this program has been evaluated by the US EPA/EC Joint Project on the Evaluation of (Q)SARs (OECD, 1994), and by Pedersen *et al.* (1995). The latter is briefly referred below.

3.8.2   A validation set of experimentally determined biodegradation data was selected among the data from MITI (1992), but excluding substances for which no precise degradation data were available and substances already used for development of the programme. The validation set then consisted of 304 substances. The biodegradability of these substances were estimated by use of the programme's non-linear estimation module (the most reliable) and the results compared with the measured data. 162 substances were predicted to degrade "fast", but only 41 (25%) were actually readily degradable in the MITI I test. 142 substances were predicted to degrade "slowly", which was confirmed by 138 (97%) substances being not readily degradable in the MITI I test. Thus, it was concluded that the programme may be used for classification purposes only when no experimental degradation data can be obtained, and when the programme predicts a substance to be degraded "slowly". In this case, the substance can be regarded as not rapidly degradable.

3.8.3   The same conclusion was reached in the US EPA/EC Joint Project on the Evaluation of (Q)SARs by use of experimental and QSAR data on new substances notified in the EU. The evaluation was based on an analysis of QSAR predictions on 115 new substances also tested experimentally in ready biodegradability tests. Only 9 of the substances included in this analysis were readily biodegradable. The employed QSAR

methodology is not fully specified in the final report of the Joint US EPA/EC project (OECD, 1994), but it is likely that the majority of predictions were made by using methods which later have been integrated in the Biodegradation Probability Program.

3.8.4   Also in the EU TGD (EC, 1996) it is recommended that estimated biodegradability by use of the Biodegradation Probability Program is used only in a conservative way, i.e. when the programme predicts fast biodegradation, this result should not be taken into consideration, whereas predictions of slow biodegradation may be considered (EC, 1996).

3.8.5   Thus, the use of results of the Biodegradability Probability Program in a conservative way may fulfil the needs for evaluating biodegradability of some of the large number of substances for which no experimental degradation data are available.

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APPENDIX II

Factors influencing degradability in the aquatic environment

1. Introduction

1.1 The OECD classification criteria are considering the hazards to the aquatic environment only. However, the hazard classification is primarily based on data prepared by conduction of tests under laboratory conditions that only seldom are similar to the conditions in the environment. Thus, the interpretation of laboratory test data for prediction of the hazards in the aquatic environment should be considered.

1.2 Interpretation of test results on biodegradability of organic substances has been considered in the OECD Detailed Review Paper on Biodegradability Testing (OECD, 1995).

1.3 The conditions in the environment are typically very different from the conditions in the standardized test systems, which make the extrapolation of degradation data from laboratory tests to the environment difficult. Among the differences, the following have significant influence on the degradability:

(a) Organism related factors (presence of competent micro-organisms);

(b) Substrate related factors (concentration of the substance and presence of other substrates); and

(c) Environment related factors (physico-chemical conditions, presence of nutrients, bioavailability of the substance).

These aspects will be discussed further below.

2. Presence of competent micro-organisms

2.1 Biodegradation in the aquatic environment is dependent on the presence of competent micro-organisms in sufficient numbers. The natural microbial communities consist of a very diverse biomass and when a 'new' substance is introduced in a sufficiently high concentration, the biomass may be adapted to degrade this substance. Frequently, the adaptation of the microbial population is caused by the growth of specific degraders that by nature are competent to degrade the substance. However, also other processes as enzyme induction, exchange of genetic material and development of tolerance to toxicity may be involved.

2.2 Adaptation takes place during a "lag" phase, which is the time period from the onset of the exposure until a significant degradation begins. It seems obvious that the length of the lag phase will depend on the initial presence of competent degraders. This will again depend on the history of the microbial community, i.e. whether the community formerly has been exposed to the substance. This means that when a xenobiotic substance has been used and emitted ubiquitously in a number of years, the likelihood of finding competent degraders will increase. This will especially be the case in environments receiving emissions as e.g. biological wastewater treatment plants. Often more consistent degradation results are found in tests where inocula from polluted waters are used compared to tests with inocula from unpolluted water (OECD, 1995; Nyholm and Ingerslev, 1997).

2.3 A number of factors determine whether the potential for adaptation in the aquatic environment is comparable with the potential in laboratory tests. Among other things adaptation depends on:

(a) initial number of competent degraders in the biomass (fraction and number);

(b) presence of surfaces for attachment;

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(c)   concentration and availability of substrate; and

(d)   presence of other substrates.

2.4   The length of the lag phase depends on the initial number of competent degraders and, for toxic substances, the survival and recovery of these. In standard ready biodegradability tests, the inoculum is sampled in sewage treatment plants. As the load with pollutants is normally higher than in the environment, both the fraction and the number of competent degraders may be higher than in the less polluted aquatic environment. It is, however, difficult to estimate how much longer the lag phase will be in the aquatic environment than in a laboratory test due to the likely lower initial number of competent degraders.

2.5   Over long periods of time, the initial concentration of competent degraders is not important as they will grow up when a suitable substrate is present in sufficient concentrations. However, if the degradability in a short period of time is of concern, the initial concentration of competent degrading micro-organisms should be considered (Scow, 1982).

2.6   The presence of flocs, aggregates and attached micro-organisms may also enhance adaptation by e.g. development of microbial niches with consortia of micro-organisms. This is of importance when considering the capability of adaptation in the diverse environments in sewage treatment plants or in sediment or soil. However, the total number of micro-organisms in ready biodegradability tests and in the aquatic environment are of the same orders of magnitude (10^4-10^8 cells/ml in ready biodegradability tests and 10^3-10^6 cells/ml or more in surface water (Scow, 1982). Thus, this factor is probably of minor importance.

2.7   When discussing the extrapolation to environmental conditions it may be valuable to discriminate between oligotrophic and eutrophic environments. Micro-organisms thriving under oligotrophic conditions are able to mineralize organic substrates at low concentrations (fractions of mg C/L), and they normally have a greater affinity for the substrate but lower growth rates and higher generation times than eutrophic organisms (OECD, 1995). Moreover, oligotrophs are unable to degrade chemicals in concentrations higher than 1 mg/l and may even be inhibited at high concentrations. Opposite to that, eutrophs require higher substrate concentrations before mineralization begins and they thrive at higher concentrations than oligotrophs. Thus, the lower threshold limit for degradation in the aquatic environment will depend on whether the microbial population is an oligotroph or an eutroph population. It is, however, not clear whether oligotrophs and eutrophs are different species or whether there is only an oligotrophic and an eutrophic way of life (OECD, 1995). Most pollutants reach the aquatic environment directly through discharge of wastewater and consequently, these recipients are mostly eutrophic.

2.8   From the above discussion it may thus be concluded that the chance of presence of competent degraders is greatest in highly exposed environments, i.e. in environments continuously receiving substances (which more frequently occurs for high production volume chemicals than for low production volume chemicals). These environments are often eutrophic and therefore, the degradation may require relatively high concentrations of substances before onset. On the other hand, in pristine waters competent species may be lacking, especially species capable of degradation of chemicals only occasionally released as low production volume chemicals.

3.   Substrate related factors

3.1   *Concentration of test substance*

3.1.1   In most laboratory tests, the test substance is applied in very high concentrations (2-100 mg/l) compared to the concentrations in the lower μg/l range that may be expected in the aquatic environment. In general, growth of micro-organisms is not supported when a substrate is present in concentrations below a threshold level of around 10 μg/l and at lower concentrations, even the energy requirement for maintenance is not met (OECD, 1995). The reason for this lower threshold level is possibly a lack of sufficient stimulus to initiate an enzymatic response (Scow, 1982). This means in general that the concentrations of many

substances in the aquatic environment are at a level where they can only hardly be the primary substrate for degrading micro-organisms.

3.1.2   Moreover, the degradation kinetics depends on substance concentration (S_0) compared with the saturation constant (K_s) as described in the Monod equation. The saturation constant is the concentration of the substrate resulting in a specific growth rate of 50% of the maximum specific growth rate. At substrate concentrations much lower than the saturation constant, which is the normal situation in most of the aquatic environment, the degradation can be described by first order or logistic kinetics (OECD, 1995). When a low density of micro-organisms (lower than 10^3-10^5 cells/ml) prevails (e.g. in oligotrophic waters), the population grows at ever decreasing rates which is typical of logistic kinetics. At a higher density of micro-organisms (e.g. in eutrophic waters), the substrate concentration is not high enough to support growth of the cells and first order kinetics apply, i.e. the degradation rate is proportional with the substance concentration. In practice, it may be impossible to distinguish between the two types of degradation kinetics due to uncertainty of the data (OECD, 1995).

3.1.3   In conclusion, substances in low concentrations (i.e. below 10 µg/l) are probably not degraded as primary substrates in the aquatic environment. At higher concentrations, readily degradable substances will probably be degraded as primary substrates in the environment at a degradation rate more or less proportional with the concentration of the substance. The degradation of substances as secondary substrates is discussed below.

3.2   *Presence of other substrates*

3.2.1   In the standard tests, the test substance is applied as the sole substrate for the micro-organisms while in the environment, a large number of other substrates are present. In natural waters, concentrations of dissolved organic carbon are often found in the range 1-10 mg C/l, i.e. up to a factor 1000 higher than a pollutant. However, much of this organic carbon is relatively persistent with an increasing fraction of persistent matter the longer the distance from the shore.

3.2.2   Bacteria in natural waters are primarily nourishing on exudates from algae. These exudates are mineralized very quickly (within minutes) demonstrating that there is a high degradation potential in the natural micro-organism communities. Thus, as micro-organisms compete for the variety of substrates in natural waters, there is a selection pressure among micro-organisms resulting in growth of opportunistic species capable of nourishing on quickly mineralized substrates, while growth of more specialized species is suppressed. Experiences from isolation of bacteria capable of degrading various xenobiotics have demonstrated that these organisms are often growing relatively slowly and survive on complex carbon sources in competition with more rapidly growing bacteria. When competent micro-organisms are present in the environment, their numbers may increase if the specific xenobiotic substrate is continuously released and reach a concentration in the environment sufficient to support growth. However, most of the organic pollutants in the aquatic environment are present in low concentrations and will only be degraded as secondary substrates not supporting growth.

3.2.3   On the other hand, the presence of quickly mineralized substrates in higher concentrations may facilitate an initial transformation of the xenobiotic molecule by co-metabolism. The co-metabolized substance may then be available for further degradation and mineralization. Thus, the presence of other substrates may increase the possibilities for a substance to be degraded.

3.2.4   It may then be concluded that the presence of a variety of substrates in natural waters and among them quickly mineralized substrates, may on the one hand cause a selection pressure suppressing growth of micro-organisms competent of degrading micro-pollutants. On the other hand it may facilitate an increased degradation by an initial co-metabolism followed by a further mineralization. The relative importance of these processes under natural conditions may vary depending on both the environmental conditions and the substance and no generalization can yet be established.

4.   Environment related factors

4.1   The environmental variables control the general microbial activity rather than specific degradation processes. However, the significance of the influence varies between different ecosystems and microbial species (Scow, 1982).

4.2   *Redox potential*

One of the most important environment related factors influencing the degradability is probably the presence of oxygen. The oxygen content and the related redox potential determines the presence of different types of micro-organisms in aquatic environments with aerobic organisms present in the water phase, in the upper layer of sediments and in parts of sewage treatment plants, and anaerobic organisms present in sediments and parts of sewage treatment plants. In most parts of the water phase, aerobic conditions are prevailing and the prediction of the biodegradability should be based on results from aerobic tests. However, in some aquatic environments the oxygen content may be very low in periods of the year due to eutrophication and the following decay of produced organic matter. In these periods, aerobic organisms will not be able to degrade the chemical, but anaerobic processes may take over if the chemical is degradable under anaerobic conditions.

4.3   *Temperature*

Another important parameter is the temperature. Most laboratory tests are performed at 20-25 °C (standard aerobic ready biodegradability tests), but anaerobic tests may be performed at 35 °C as this better mimics the conditions in a sludge reactor. Microbial activity is found in the environment at temperatures ranging from below 0 °C to 100 °C. However, optimum temperatures are probably in the range from 10 °C to 30 °C and roughly, the degradation rate doubles for every 10 °C increase of temperature in this range (de Henau, 1993). Outside this optimum range the activity of the degraders is reduced drastically although some specialized species (termo- and psycrophilic bacteria) may thrive. When extrapolating from laboratory conditions, it should be considered that some aquatic environments are covered by ice in substantial periods of the year and that only minor or even no degradation can be expected during the winter season.

4.4   *pH*

Active micro-organisms are found in the entire pH range found in the environment. However, for bacteria as a group, slightly alkaline conditions favour the activity and the optimum pH range is 6-8. At a pH lower than 5, the metabolic activity in bacteria is significantly decreased. For fungi as a group, slightly acidic conditions favour the activity with an optimum pH range of 5-6 (Scow, 1982). Thus, an optimum for the degrading activity of micro-organisms will probably be within the pH range of 5-8, which is the range most often prevailing in the aquatic environment.

4.5   *Presence of nutrients*

The presence of inorganic nutrients (nitrogen and phosphorus) is often required for microbial growth. However, these are only seldom the activity limiting factors in the aquatic environment where growth of micro-organisms is often substrate limited. However, the presence of nutrient influences the growth of primary producers and then again the availability of readily mineralized exudates.

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APPENDIX III

**Basic principles of the experimental and estimation methods for
determination of BCF and K_{ow} of organic substances**

1.   Bioconcentration factor (BCF)

1.1   *Definition*

The bioconcentration factor is defined as the ratio between the concentration of the chemical in biota and the concentration in the surrounding medium, here water, at steady state. BCF can be measured experimentally directly under steady-state conditions or calculated by the ratio of the first-order uptake and elimination rate constants, a method that does not require equilibrium conditions.

1.2   *Appropriate methods for experimental determination of BCF*

1.2.1   Different test guidelines for the experimental determination of bioconcentration in fish have been documented and adopted; the most generally applied being the OECD test guideline (OECD 305, 1996) and the ASTM standard guide (ASTM E 1022-94). OECD 305 (1996) was revised and replaced the previous version OECD 305A-E, (1981). Although flow-through test regimes are preferred (OECD 305, 1996), semi-static regimes are allowed (ASTM E 1022-94), provided that the validity criteria on mortality and maintenance of test conditions are fulfilled. For lipophilic substances (log $K_{ow} > 3$), flow-through methods are preferred.

1.2.2   The principles of the OECD 305 and the ASTM guidelines are similar, but the experimental conditions described are different, especially concerning:

(a)   method of test water supply (static, semi-static or flow through);

(b)   the requirement for carrying out a depuration study;

(c)   the mathematical method for calculating BCF;

(d)   sampling frequency: Number of measurements in water and number of samples of fish;

(e)   requirement for measuring the lipid content of the fish;

(f)   the minimum duration of the uptake phase;

1.2.3   In general, the test consists of two phases: The exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. A 28-day exposure phase is obligatory unless a steady state has been reached within this period. The time needed for reaching steady-state conditions may be set on the basis of $K_{ow} - k_2$ correlations (e.g. log $k_2 = 1.47 - 0.41$ log K_{ow} (Spacie and Hamelink, 1982) or log $k_2 = 1.69 - 0.53$ log K_{ow} (Gobas *et al.*, 1989)). The expected time (d) for e.g. 95% steady state may thus be calculated by: - ln(1-0.95)/k_2, provided that the bioconcentration follows first order kinetics. During the depuration phase the fish are transferred to a medium free of the test substance. The concentration of the test substance in the fish is followed through both phases of the test. The BCF is expressed as a function of the total wet weight of the fish. As for many organic substances, there is a significant relationship between the potential for bioconcentration and the lipophilicity, and furthermore, there is a corresponding relationship between the lipid content of the test fish and the observed bioconcentration of such substances. Therefore, to reduce this source of variability in the test results for the substances with high lipophilicity, bioconcentration should be expressed in relation to the lipid content in addition to whole body weight (OECD 305 (1996), ECETOC (1995)). The guidelines mentioned are based on the assumption that bioconcentration may be approximated by a first-order process (one-compartment model) and thus that BCF = k_1/k_2 (k_1: first-order uptake rate, k_2:

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first-order depuration rate, described by a log-linear approximation). If the depuration follows biphasic kinetics, i.e. two distinct depuration rates can be identified, the approximation k_1/k_2 may significantly underestimate BCF. If a second order kinetic has been indicated, BCF may be estimated from the relation: C_{Fish}/C_{Water}, provided that "steady-state" for the fish-water system has been reached.

1.2.4   Together with details of sample preparation and storage, an appropriate analytical method of known accuracy, precision, and sensitivity must be available for the quantification of the substance in the test solution and in the biological material. If these are lacking it is impossible to determine a true BCF. The use of radiolabelled test substance can facilitate the analysis of water and fish samples. However, unless combined with a specific analytical method, the total radioactivity measurements potentially reflect the presence of parent substance, possible metabolite(s), and possible metabolized carbon, which have been incorporated in the fish tissue in organic molecules. For the determination of a true BCF it is essential to clearly discriminate the parent substance from possible metabolites. If radiolabelled materials are used in the test, it is possible to analyse for total radio label (i.e. parent and metabolites) or the samples may be purified so that the parent compound can be analysed separately.

1.2.5   In the log K_{ow} range above 6, the measured BCF data tend to decrease with increasing log K_{ow}. Conceptual explanations of non-linearity mainly refer to either biotransformation, reduced membrane permeation kinetics or reduced biotic lipid solubility for large molecules. Other factors consider experimental artefacts, such as equilibrium not being reached, reduced bioavailability due to sorption to organic matter in the aqueous phase, and analytical errors. Moreover, care should be taken when evaluating experimental data on BCF for substances with log K_{ow} above 6, as these data will have a much higher level of uncertainty than BCF values determined for substances with log K_{ow} below 6.

2.   log K_{ow}

2.1   *Definition and general considerations*

2.1.1   The log *n*-octanol-water partition coefficient (log K_{ow}) is a measure of the lipophilicity of a substance. As such, log K_{ow} is a key parameter in the assessment of environmental fate. Many distribution processes are driven by log K_{ow}, e.g. sorption to soil and sediment and bioconcentration in organisms.

2.1.2   The basis for the relationship between bioconcentration and log K_{ow} is the analogy for the partition process between the lipid phase of fish and water and the partition process between n-octanol and water. The reason for using K_{ow} arises from the ability of octanol to act as a satisfactory surrogate for lipids in fish tissue. Highly significant relationships between log K_{ow} and the solubility of substances in cod liver oil and triolin exist (Niimi, 1991). Triolin is one of the most abundant triacylglycerols found in freshwater fish lipids (Henderson and Tocher, 1987).

2.1.3   The determination of the *n*-octanol-water partition coefficient (K_{ow}) is a requirement of the base data set to be submitted for notified new and priority existing substances within the EU. As the experimental determination of the K_{ow} is not always possible, e.g. for very water-soluble and for very lipophilic substances, a QSAR derived K_{ow} may be used. However, extreme caution should be exercized when using QSARs for substances where the experimental determination is not possible (as for e.g. surfactants).

2.2   *Appropriate methods for experimental determination of K_{ow} values*

2.2.1   For experimental determination of K_{ow} values, two different methods, Shake-flask and HPLC, have been described in standard guidelines e.g. OECD 107 (1995); OECD 117 (1983); EEC A.8. (1992); EPA-OTS (1982); EPA-FIFRA (1982); ASTM (1993). Not only data obtained by the employment of the shake-flask or the HPLC method according to standard guidelines are recommended. For highly lipophilic substances, which are slowly soluble in water, data obtained by employing a slow-stirring method are generally more reliable (De Bruijn *et al*., 1989; Tolls and Sijm, 1993; OECD draft Guideline, 1998). The slow stirring method is currently being ringtested for development of a final OECD guideline.

2.2.2   *Shake-flask method*

The basic principle of the method is to measure the dissolution of the substance in two different phases, water and *n*-octanol. In order to determine the partition coefficient, equilibrium between all interacting components of the system must be achieved after which the concentration of the substances dissolved in the two phases is determined. The shake-flask method is applicable when the log K_{ow} value falls within the range from -2 to 4 (OECD 107, 1995). The shake-flask method applies only to essential pure substances soluble in water and *n*-octanol and should be performed at a constant temperature ($\pm1°C$) in the range 20-25 °C.

2.2.3   *HPLC method*

HPLC is performed on analytical columns packed with a commercially available solid phase containing long hydrocarbon chains (e.g. C_8, C_{18}) chemically bound onto silica. Chemicals injected onto such a column move along at different rates because of the different degrees of partitioning between the mobile aqueous phase and the stationary hydrocarbon phase. The HPLC method is not applicable to strong acids and bases, metals complexes, surface-active materials, or substances that react with the eluent. The HPLC method is applicable when the log K_{ow} value falls within the range 0 to 6 (OECD 117, 1989). The HPLC method is less sensitive to the presence of impurities in the test compound compared to the shake-flask method.

2.2.4   *Slow stirring method*

With the slow-stirring method a precise and accurate determination of K_{ow} of compounds with log K_{ow} up till 8.2 is allowed (De Bruijn *et al*., 1989). For highly lipophilic compounds the shake-flask method is prone to produce artefacts (formation of microdroplets), and with the HPLC method K_{ow} needs to be extrapolated beyond the calibration range to obtain estimates of K_{ow}.

In order to determine a partition coefficient, water, n-octanol, and test compound are equilibrated with each other after which the concentration of the test compound in the two phases is determined. The experimental difficulties associated with the formation of microdroplets during the shake-flask experiment can to some degree be overcome in the slow-stirring experiment as water, octanol, and the test compound are equilibrated in a gently stirred reactor. The stirring creates a more or less laminar flow between the octanol and the water, and exchange between the phases is enhanced without microdroplets being formed.

2.2.5   *Generator column method*

Another very versatile method for measuring log K_{ow} is the generator column method. In this method, a generator column method is used to partition the test substance between the octanol and water phases. The column is packed with a solid support and is saturated with a fixed concentration of the test substance in *n*-octanol. The test substance is eluted from the octanol -saturated generator column with water. The aqueous solution exiting the column represents the equilibrium concentration of the test substance that has partitioned from the octanol phase into the water phase. The primary advantage of the generator column method over the shake flask method is that the former completely avoids the formation of micro-emulsions. Therefore, this method is particularly useful for measuring K_{ow} for substances values over 4.5 (Doucette and Andren, 1987 and 1988; Shiu *et al.*, 1988) as well as for substances having log K_{ow} values less than 4.5. A disadvantage of the generator column method is that it requires sophisticated equipment. A detailed description of the generator column method is presented in the "Toxic Substances Control Act Test Guidelines" (USEPA 1985).

2.3   *Use of QSARs for determination of log K_{ow} (see also in A9.6, « Use of QSARs »)*

2.3.1   Numerous QSARs have been and continue to be developed for the estimation of K_{ow}. Commonly used methods are based on fragment constants. The fragmental approaches are based on a simple addition of the lipophilicity of the individual molecular fragments of a given molecule. Three commercially available PC

programs are recommended in the European Commission's Technical Guidance Document (European Commission, 1996) for risk assessment, part III, if no experimentally derived data are available.

2.3.2   CLOGP (Daylight Chemical Information Systems, 1995) was initially developed for use in drug design. The model is based on the Hansch and Leo calculation procedure (Hansch and Leo, 1979). The program calculates log K_{ow} for organic compounds containing C, H, N, O, Hal, P, and/or S. Log K_{ow} for salts and for compounds with formal charges cannot be calculated (except for nitro compounds and nitrogen oxides). The calculation results of log K_{ow} for ionizable substances, like phenols, amines, and carboxylic acids, represent the neutral or unionized form and will be pH dependent. In general, the program results in clear estimates in the range of log Kow between 0 and 5 (European Commission, 1996, part III). However a validation study performed by Niemelä (1993), who compared experimental determined log K_{ow} values with estimated values, showed that the program precisely predicts the log K_{ow} for a great number of organic chemicals in the log K_{ow} range from below 0 to above 9 (n = 501, r^2 = 0.967). In a similar validation study on more than 7000 substances the results with the CLOGP-program (PC version 3.32, EPA version 1.2) were r^2 = 0.89, s.d.= 0.58, n = 7221. These validations show that the CLOGP-program may be used for estimating reliable log K_{ow} values when no experimental data are available. For chelating compounds and surfactants the CLOGP program is stated to be of limited reliability (OECD, 1993). However, as regards anionic surfactants (LAS) a correction method for estimating adjusted CLOGP values has been proposed (Roberts, 1989).

2.3.3   LOGKOW or KOWWIN (Syracuse Research Corporation) uses structural fragments and correction factors. The program calculates log K_{ow} for organic compounds containing the following atoms: C, H, N, O, Hal, Si, P, Se, Li, Na, K, and/or Hg. Log K_{ow} for compounds with formal charges (like nitrogenoxides and nitro compounds) can also be calculated. The calculation of log K_{ow} for ionizable substances, like phenols, amines and carboxylic acids, represent the neutral or unionized form, and the values will thus be pH dependent. Some surfactants (e.g. alcohol ethoxylates (Tolls, 1998), dyestuffs, and dissociated substances may be predicted by the LOGKOW program (Pedersen *et al*, 1995). In general, the program gives clear estimates in the range of log K_{ow} between 0 and 9 (TemaNord 1995:581). Like the CLOGP-program, LOGKOW has been validated (Table 2) and is recommended for classification purposes because of its reliability, commercial availability, and convenience of use.

2.3.4   AUTOLOGP (Devillers *et al*., 1995) has been derived from a heterogeneous data set, comprising 800 organic chemicals collected from literature. The program calculates log K_{ow} values for organic chemicals containing C, H, N, O, Hal, P, and S. The log K_{ow} values of salts cannot be calculated. Also the log K_{ow} of some compounds with formal charges cannot be calculated, with the exception of nitro compounds. The log K_{ow} values of ionizable chemicals like phenols, amines, and corboxylic acids can be calculated although pH-dependencies should be noted. Improvements are in progress in order to extend the applicability of AUTOLOGP. According to the presently available information, AUTOLOGP gives accurate values especially for highly lipophilic substances (log K_{ow} > 5) (European Commission, 1996).

2.3.5   SPARC. The SPARC model is still under development by EPA's Environmental Research Laboratory in Athens, Georgia, and is not yet public available. SPARC is a mechanistic model based on chemical thermodynamic principles rather than a deterministic model rooted in knowledge obtained from observational data. Therefore, SPARC differs from models that use QSARs (i.e. KOWWIN, LOGP) in that no measured log K_{ow} data are needed for a training set of chemicals. EPA does occasionally run the model for a list of CAS numbers, if requested. SPARC provides improved results over KOWWIN and CLOGP only for compounds with log K_{ow} values greater than 5. Only SPARC can be employed in a general way for inorganic or organometallic compounds.

In Table 1, this Appendix, an overview of log K_{ow} estimation methods based on fragmentation methodologies is presented. Also other methods for the estimation of log K_{ow} values exist, but they should only be used on a case-by-case basis and only with appropriate scientific justification.

Table 1: Overview of QSAR methods for estimation of log K_{ow} based on fragmentation methodologies (Howard and Meylan (1997))

Method	Methodology	Statistics
CLOGP Hansch and Leo (1979), CLOGP Daylight (1995)	Fragments + correction factors	Total n = 8942, r^2= 0,917, sd = 0,482 Validation: n = 501, r^2 = 0,967 Validation: n = 7221, r^2 = 0,89, sd = 0,58
LOGKOW (KOWWIN) Meylan and Howard (1995), SRC	140 fragments 260 correction factors	Calibration: n = 2430, r^2= 0,981, sd = 0,219, me = 0,161 Validation: n = 8855, r^2= 0,95, sd = 0,427, me = 0,327
AUTOLOGP Devillers *et al.* (1995)	66 atomic and group contributions from Rekker and Manhold (1992)	Calibration: n = 800, r^2= 0,96, sd = 0,387
SPARC Under development by EPA, Athens, Georgia.	Based upon fundamental chemical structure algorithm.	No measured log Kow data are needed for a training set of chemicals.
Rekker and De Kort (1979)	Fragments + correction factors	Calibration n = 1054, r^2 = 0,99 Validation: n = 20, r^2 = 0,917, sd = 0,53, me = 0,40
Niemi *et al.* (1992)	MCI	Calibration n = 2039, r^2 = 0,77 Validation: n = 2039, r^2 = 0,49
Klopman *et al* (1994)	98 fragments + correction factors	Calibration n = 1663, r^2 = 0,928, sd = 0,3817
Suzuki and Kudo (1990)	424 fragments	Total: n= 1686, me = 0,35 Validation: n = 221, me = 0,49
Ghose *et al.* (1988) ATOMLOGP	110 fragments	Calibration: n = 830, r^2= 0,93, sd = 0,47 Validation: n = 125, r^2= 0,87, sd = 0,52
Bodor and Huang (1992)	Molecule orbital	Calibration: n = 302, r^2= 0,96, sd = 0,31, me = 0,24 Validation: n = 128, sd = 0,38
Broto *et al.* (1984) ProLogP	110 fragments	Calibration: n = 1868, me= ca. 0,4

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APPENDIX IV

Influence of external and internal factors on the bioconcentration potential of organic substances

1.   Factors influencing the uptake

The uptake rate for lipophilic compounds is mainly a function of the size of the organism (Sijm and Linde, 1995). External factors such as the molecular size, factors influencing the bioavailability, and different environmental factors are of great importance to the uptake rate as well.

1.1   *Size of organism*

Since larger fish have a relatively lower gill surface to weight ratio, a lower uptake rate constant (k_1) is to be expected for large fish compared to small fish (Sijm and Linde, 1995; Opperhuizen and Sijm, 1990). The uptake of substances in fish is further controlled by the water flow through the gills; the diffusion through aqueous diffusion layers at the gill epithelium; the permeation through the gill epithelium; the rate of blood flow through the gills, and the binding capacity of blood constituents (ECETOC, 1995).

1.2   *Molecular size*

Ionized substances do not readily penetrate membranes; as aqueous pH can influence the substance uptake. Loss of membrane permeability is expected for substances with a considerable cross-sectional area (Opperhuizen *et al*., 1985; Anliker *et al*., 1988) or long chain length (> 4.3 nm) (Opperhuizen, 1986). Loss of membrane permeability due to the size of the molecules will thus result in total loss of uptake. The effect of molecular weight on bioconcentration is due to an influence on the diffusion coefficient of the substance, which reduces the uptake rate constants (Gobas *et al*., 1986).

1.3   *Availability*

Before a substance is able to bioconcentrate in an organism it needs to be present in water and available for transfer across fish gills. Factors, which affect this availability under both natural and test conditions, will alter the actual bioconcentration in comparison to the estimated value for BCF. As fish are fed during bioconcentration studies, relatively high concentrations of dissolved and particulate organic matter may be expected, thus reducing the fraction of chemical that is actually available for direct uptake via the gills. McCarthy and Jimenez (1985) have shown that adsorption of lipophilic substances to dissolved humic materials reduces the availability of the substance, the more lipophilic the substance the larger reduction in availability (Schrap and Opperhuizen, 1990). Furthermore, adsorption to dissolved or particulate organic matter or surfaces in general may interfere during the measurement of BCF (and other physical-chemical properties) and thus make the determination of BCF or appropriate descriptors difficult. As bioconcentration in fish is directly correlated with the available fraction of the chemical in water, it is necessary for highly lipophilic substances to keep the available concentration of the test chemical within relatively narrow limits during the uptake period.

Substances, which are readily biodegradable, may only be present in the test water for a short period, and bioconcentration of these substances may thus be insignificant. Similarly, volatility and hydrolysis will reduce the concentration and time in which the substance is available for bioconcentration.

1.4   *Environmental factors*

Environmental parameters influencing the physiology of the organism may also affect the uptake of substances. For instance, when the oxygen content of the water is lowered, fish have to pass more water over their gills in order to meet respiratory demands (McKim and Goeden, 1982). However, there may be species dependency as indicated by Opperhuizen and Schrap (1987). It has, furthermore, been shown that the

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temperature may have an influence on the uptake rate constant for lipophilic substances (Sijm *et al*. 1993), whereas other authors have not found any consistent effect of temperature changes (Black *et al*. 1991).

2.   Factors influencing the elimination rate

The elimination rate is mainly a function of the size of the organism, the lipid content, the biotransformation process of the organism, and the lipophilicity of the test compound.

2.1   *Size of organism*

As for the uptake rate the elimination rate is dependent on the size of the organism. Due to the higher gill surface to weight ratio for small organisms (e.g. fish larvae) than that of large organisms, steady-state and thus "toxic dose equilibrium" has shown to be reached sooner in early life stages than in juvenile/adult stages of fish (Petersen and Kristensen, 1998). As the time needed to reach steady-state conditions is dependent on k_2, the size of fish used in bioconcentration studies has thus an important bearing on the time required for obtaining steady-state conditions.

2.2   *Lipid content*

Due to partitioning relationships, organisms with a high fat content tend to accumulate higher concentrations of lipophilic substances than lean organisms under steady-state conditions. Body burdens are therefore often higher for "fatty" fish such as eel, compared to "lean" fish such as cod. In addition, lipid "pools" may act as storage of highly lipophilic substances. Starvation or other physiological changes may change the lipid balance and release such substances and result in delayed impacts.

2.3   *Metabolism*

2.3.1   In general, metabolism or biotransformation leads to the conversion of the parent compound into more water-soluble metabolites. As a result, the more hydrophilic metabolites may be more easily excreted from the body than the parent compound. When the chemical structure of a compound is altered, many properties of the compound are altered as well. Consequently the metabolites will behave differently within the organism with respect to tissue distribution, bioaccumulation, persistence, and route and rate of excretion. Biotransformation may also alter the toxicity of a compound. This change in toxicity may either be beneficial or harmful to the organism. Biotransformation may prevent the concentration in the organism from becoming so high that a toxic response is expressed (detoxification). However, a metabolite may be formed which is more toxic than the parent compound (bioactivation) as known for e.g. benzo(a)pyrene.

2.3.2   Terrestrial organisms have a developed biotransformation system, which is generally better than that of organisms living in the aquatic environment. The reason for this difference may be the fact that biotransformation of xenobiotics may be of minor importance in gill breathing organisms as they can relatively easily excrete the compound into the water (Van Den Berg *et al*. 1995). Concerning the biotransformation capacity in aquatic organisms the capacity for biotransformation of xenobiotics increases in general as follows: Molluscs < crustaceans < fish (Wofford *et al*., 1981).

3.   Lipophilicity of substance

A negative linear correlation between k_2 (depuration constant) and log K_{ow} (or BCF) has been shown in fish by several authors (e.g. Spacie and Hamelink, 1982; Gobas *et al*., 1989; Petersen and Kristensen, 1998), whereas k_1 (uptake rate constant) is more or less independent of the lipophilicity of the substance (Connell, 1990). The resultant BCF will thus generally increase with increasing lipophilicity of the substances, i.e. log BCF and log K_{ow} correlate for substances which do not undergo extensive metabolism.

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APPENDIX V

Test guidelines

1.   Most of the guidelines mentioned are found in compilations from the organisation issuing them. The main references to these are:

(a) EC guidelines: European Commission (1996). Classification, Packaging and Labelling of Dangerous Substances in the European Union. Part 2 – Testing Methods. European Commission. 1997. ISBN92-828-0076-8. (Homepage: http://ecb.ei.jrc.it/testing-methods/);

(b) ISO guidelines: Available from the national standardisation organisations or ISO (Homepage: http://www.iso.ch/);

(c) OECD guidelines for the testing of chemicals. OECD, Paris, 1993 with regular updates (Homepage: http://www.oecd.org/ehs/test/testlist.htm);

(d) OPPTS guidelines: US-EPA homepage:http://www.epa.gov/opptsfrs/home/guidelin.htm and (http://www.epa.gov/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines / Drafts);

(e) ASTM: ASTM's homepage: http://www.astm.org. Further search via "standards".

2.   Test guidelines for aquatic toxicity [1]

OECD Test Guideline 201 (1984) Alga, Growth Inhibition Test
OECD Test Guideline 202 (1984) Daphnia sp. Acute Immobilisation Test and Reproduction Test
OECD Test Guideline 203 (1992) Fish, Acute Toxicity Test
OECD Test Guideline 204 (1984) Fish, Prolonged Toxicity Test: 14-Day Study
OECD Test Guideline 210 (1992) Fish, Early-Life Stage Toxicity Test
OECD Test Guideline 211 (1998) Daphnia magna Reproduction Test
OECD Test Guideline 212 (1998) Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages
OECD Test Guideline 215 (2000) Fish, Juvenile Growth Test
OECD Test Guideline 221 (in preparation) *Lemna* sp. Growth inhibition test
EC C.1: Acute Toxicity for Fish (1992)
EC C.2: Acute Toxicity for Daphnia (1992)
EC C.3: Algal Inhibition Test (1992)
EC C.14: Fish Juvenile Growth Test (2001)
EC C.15: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages (2001)
EC C.20: Daphnia Magna Reproduction Test (2001)

OPPTS Testing Guidelines for Environmental Effects (850 Series Public Drafts):

850.1000 Special consideration for conducting aquatic laboratory studies
850.1000 Special consideration for conducting aquatic laboratory studies
850.1010 Aquatic invertebrate acute toxicity, test, freshwater daphnids
850.1010 Aquatic invertebrate acute toxicity, test, freshwater daphnids
850.1020 Gammarid acute toxicity test

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[1]   *The list below is as of September 2000 and will need to be regularly updated as new guidelines are adopted or draft guidelines are elaborated.*

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850.1020 Gammarid acute toxicity test

850.1035 Mysid acute toxicity test

850.1035 Mysid acute toxicity test

850.1045 Penaeid acute toxicity test

850.1045 Penaeid acute toxicity test

850.1075 Fish acute toxicity test, freshwater and marine

850.1075 Fish acute toxicity test, freshwater and marine

850.1300 Daphnid chronic toxicity test

850.1300 Daphnid chronic toxicity test

850.1350 Mysid chronic toxicity test

850.1350 Mysid chronic toxicity test

850.1400 Fish early-life stage toxicity test

850.1400 Fish early-life stage toxicity test

850.1500 Fish life cycle toxicity

850.1500 Fish life cycle toxicity

850.1730 Fish BCF

850.1730 Fish BCF

850.4400 Aquatic plant toxicity test using Lemna spp. Tiers I and II

850.4400 Aquatic plant toxicity test using Lemna spp. Tiers I and II

850.4450 Aquatic plants field study, Tier III

850.4450 Aquatic plants field study, Tier III

850.5400 Algal toxicity, Tiers I and II

850.5400 Algal toxicity, Tiers I and II

3.   Test guidelines for biotic and abiotic degradation [2]

ASTM E 1196-92

ASTM E 1279-89(95) Standard test method for biodegradation by a shake-flask die-away method

ASTM E 1625-94 Standard test method for determining biodegradability of organic chemicals in semi-continuous activated sludge (SCAS)

EC C.4. A to F: Determination of ready biodegradability. Directive 67/548/EEC, Annex V. (1992)

EC C.5. Degradation: biochemical oxygen demand. Directive 67/548/EEC, Annex V. (1992)

EC C.7. Degradation: abiotic degradation: hydrolysis as a function of pH. Directive 67/548/EEC, Annex V. (1992)

EC C.9. Biodegradation: Zahn-Wellens test. Directive 67/548/EEC, Annex V. (1988)

EC C.10. Biodegradation: Activated sludge simulation tests. Directive 67/548/EEC, Annex V. (1998)

EC C.11. Biodegradation: Activated sludge respiration inhibition test. Directive 67/548/EEC, AnnexV.(1988)

EC C.12. Biodegradation: Modified SCAS test. Directive 67/548/EEC, Annex V. (1998)

ISO 9408 (1991). Water quality - Evaluation in an aqueous medium of the "ultimate" biodegradability of organic compounds - Method by determining the oxygen demand in a closed respirometer

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[2]   *The list below is as of September 2000 and will need to be regularly updated as new guidelines are adopted or draft guidelines are elaborated.*

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ISO 9888 (1991). Water quality - Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium - Static test (Zahn-Wellens method)

ISO 10707 (1994). Water quality - Evaluation in an aqueous medium of the "ultimate" biodegradability of organic compounds - Method by analysis of biochemical oxygen demand (closed bottle test)

ISO 11348 (1997). Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)

ISO 11733 (1994). Water quality - Evaluation of the elimination and biodegradability of organic compounds in an aqueous medium - Activated sludge simulation test

ISO 11734 (1995). Water quality - Evaluation of the "ultimate" anaerobic biodegradability of organic compounds in digested sludge - Method by measurement of the biogas production

ISO/DIS 14592 .(1999) Water quality - Evaluation of the aerobic biodegradability of organic compounds at low concentrations in water. Part 1: Shake flask batch test with surface water or surface water/sediment suspensions (22.11.1999)

OECD Test Guideline 111 (1981). Hydrolysis as a function of pH. OECD guidelines for testing of chemicals

OECD Test Guideline 209 (1984). Activated sludge, respiration inhibition test. OECD guidelines for testing of chemicals

OECD Test Guideline 301 (1992). Ready biodegradability. OECD guidelines for testing of chemicals

OECD Test Guideline 302A (1981). Inherent biodegradability: Modified SCAS test. OECD guidelines for testing of chemicals

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OECD Test Guideline 306 (1992). Biodegradability in seawater. OECD guidelines for testing of chemicals

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OECD (1999). Aerobic and anaerobic transformation in soil. Final text of a draft proposal for a new guideline, October. 1999

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OPPTS 835.2110 Hydrolysis as a function of pH

OPPTS 835.2130 Hydrolysis as a function of pH and temperature

OPPTS 835.2210 Direct photolysis rate in water by sunlight

OPPTS 835.3110 Ready biodegradability

OPPTS 835.3170 Shake flask die-away test

OPPTS 835.3180 Sediment/water microcosm biodegradability test

OPPTS 835.3200 Zahn-Wellens/EMPA test

OPPTS 835.3210 Modified SCAS test

OPPTS 835.3300 Soil biodegradation

OPPTS 835.3400 Anaerobic biodegradability of organic chemicals

OPPTS 835.5270 Indirect photolysis screening test: Sunlight photolysis in waters containing dissolved humic substances

4. Test guidelines for bioaccumulation [3]

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[3] *The list below is as of September 2000 and will need to be regularly updated as new guidelines are adopted or draft guidelines are elaborated.*

Annex 9

APPENDIX VI

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